CHONDROGENIC DIFFERENTIATION OF HUMAN ADIPOSE DERIVED STEM CELLS: THE ROLES OF MECHANICAL LOADING, ELEVATED CALCIUM, AND THE EXTRACELLULAR CALCIUM SENSING RECEPTOR.

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ABSTRACT

JOHN MICHAEL WILLIAMS II: Chondrogenic Differentiation of Human Adipose Derived Stem Cells: The Roles of Mechanical Loading, Elevated Calcium, and the Calcium Sensing Receptor. (Under the direction of Elizabeth G. Loboa).

Cartilage defects caused by injury or disease affect millions each year. Human adipose derived stem cells, hASC, have emerged as an opportune cell source for cartilage tissue engineering. This research first aims to observe the optimal conditions to direct chondrogenic differentiation, focusing on the role of mechanical loading, through a review of the literature. Because of Ca^{2+} affects in both chemical and mechanical signaling, hASC were pellet cultured in chondrogenic differentiation media with elevated Ca^{2+} . These pellets demonstrated diminished chondrogenesis, and a layer of calcified, hypertrophic cartilage. To better understand the mechanism by which Ca^{2+} hASC were cultured with either transient knockdown of Polycystin, PC2, or allosteric binding of the extracellular calcium sensing receptor (CaR) in elevated Ca^{2+}. Modulating the CaR affects the impact of elevated Ca^{2+} in hASC differentiation. The use of Ca^{2+} and its various mechanisms for cell signaling will prove to be a valuable tool in developing novel techniques for tissue engineering.

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List of Abbreviations and Symbols

ALP-Alkaline phosphatase

BMP6- Bone morphogenetic protein 6

Ca^{2+}-Calcium ions

CaR- Extracellular calcium sensing receptor

CDM-Chondrogenic differentiation media

CGM-Complete growth media

CHP-Cyclic hydrostatic pressure

Col-Collagen

DNA-Deoxyribonucleic acid

FBS-Fetal bovine serum

GAG-Glycosaminoglycan

hASC- Human adipose derived stem cells

hMSC-Human bone-marrow derived mesenchymal stem cells

ITS+- Insulin, transferrin, selenous acid

MMP-Matrix metalloproteinase

mRNA-messenger Ribonucleic acid

PC2- Polycystin 2

PCR-Polymerase chain reaction

siRNA-small interfering ribonucleic acid

SPP1- Ostepontin

Chapter 1:

Introduction

1.1 Motivation

Cartilage is an avascular, connective tissue with the critical function of supporting movement. The tissue is responsible for bearing multiples types of load repetitively, without degrading. Further, because of its avascular nature, the tissue has little regenerative capability. Cartilage defects can come about due to injury, disease, or the aging process, and can lead to reduced mobility and quality of life. While there are a number of methods of adjusting to minimize the impact of the damaged tissue, few treatments exist to effectively repair defective cartilage tissue. The Cell Mechanics Laboratory specializes in understanding the mechanical forces necessary to engineer functional musculoskeletal tissue from mesenchymal stem cells derived from both bone marrow and adipose tissue. it is our goal to develop novel techniques for cartilage tissue engineering utilizing hASC.

Some concerns with current treatments for cartilage defect repair are the integration of engineered tissue into the joint, as well as the material properties of the engineered tissue. We plan to address the issue of assimilation by the using an autologous cell source, limiting the body's immune response. Also, mechanical loading plays a key role in native cartilage tissue metabolism and homeostasis. We plan to leverage this to condition engineered tissue with loading protocols comparable to those seen in vivo, ensuring the tissue can properly bear the expected loads.

In order to optimize cartilage tissue engineering, we will need to better understand the physical environment as well as the chemical environment, as it changes during mechanical loading. This study will also work to better understand the mechanisms by which these forces direct differentiation.

1.2 Thesis Findings

The findings of this body of work have been shared in numerous professional academic conferences and are being submitted to peer-reviewed journals for publication.

Manuscripts in preparation:

The Role of Mechanical Loading in Cartilage Tissue Engineering with Human Adipose Derived Stem Cells

Williams, JM; Loboa EG

Planned journal: *Tissue Engineering, Pt B*

Elevated Extracellular Calcium Inhibits Chondrogenic Differentiation of hASC

Williams, JM; Kannan A; Dent MR; Estes B; Moutos F, Hluck BH, Guilack F,; Loboa EG

Planned journal: *Tissue Engineering, Pt A*

Targeting the Extracellular Calcium Sensing Receptor in Human Adipose Derived Stem Cell Osteogenic Differentiation

Williams JM; Bodle JC; Loboa EG

Planned journal: *Journal of Bone and Mineral Research*

Conference Proceedings

Elevated Extracellular Calcium Inhibits Chondrogenic Differentiation of Human Adipose Derived Stem Cells in Pellet Culture

Williams JM; Kannan A; Dent MR; Estes B; Moutos F; Hluck BH; Bernacki SH; Guilak F; Loboa EG

Presented at NCTERMS 2011, Winston Salem NC

Chapter 2:

Literature Review

2.1 Human Adipose Derived Stem Cells and Chondrogenesis

Human adipose-derived stem cells (hASC) show great potential for cartilage tissue engineering applications. When exposed to appropriate chemical and mechanical stimuli, these cells provide a number of distinct advantages over previously studied cells while behaving in a comparable manner with respect to chondrogenic potential. These cells are relatively more abundant than either bone marrow derived mesenchymal stem cells, hMSC, or articular chondrocytes, with less donor site morbidity, and greater expansion capabilities.[1-6] A number of chemical chondrogenic induction protocols previously used with other stem and progenitor cells have been adapted for use with hASC in cartilage tissue engineering, with varying levels of success[7-13]. Some chondrogenic conditions, such as soluble growth factors, 3D culture, low oxygen concentration, and mechanical loading, have proven necessary for successful cartilage tissue engineering regardless of the cell source [14-31]. However, further study of the optimal conditions, in particular, the less understood role of mechanical stimulation for induction of hASC chondrogenesis, must continue in order to achieve long term success of engineered cartilage tissue. This review focuses on current knowledge in the published literature on the effects of mechanical stimuli on chondrogenic differentiation of hASC, specifically, hydrostatic pressure, compression, tensile strain and shear stress.

2.2 Cartilage tissue

Cartilage is one of the most critical and complex musculoskeletal materials within the body. When healthy it provides a number of key functions, varying from providing the structure for facial features as in the ears and nose, to providing critical mechanical buffering between bones at joints. The complex nature of the material provides it with the mechanical properties to perform each of these functions. Unfortunately, the same complexity and functionality that are crucial to cartilage function, has made repair and/or regeneration of defective or damaged cartilage that much more difficult. Degenerative conditions such as rheumatoid and osteoarthritis, which affect millions of people, along with common injuries, have demonstrated that damaged cartilage not only greatly diminishes mobility, but also quality of life [32-34].

One of the major issues with cartilage defects or injuries is the inability of articular cartilage to regenerate naturally. Articular cartilage tissue is avascular, leaving the material no access to many of the regenerative nutrients found within blood. When a vascular supply is present during *in vivo* chondrogenesis, as in wound or fracture healing, the resulting cartilage is usually a type of loosely organized fibrocartilage associated with wound healing, which possesses significantly different mechanical properties than articular cartilage, and is less functionally capable [35-37]. Additionally, the regenerative capabilities of the sparse community of living cells within the tissue are limited. The chondrocyte, which produces the extracellular matrix (ECM) of cartilage, maintains a very low level of metabolic activity, and therefore limited turnover of tissue [32,38]. While chondrocytes are able to maintain a level of anabolic/catabolic remodeling of the ECM in response to mechanical loading and cytokine signaling, these responses are limited [38-40]. Additionally, increased proliferation and catabolic activities have been associated with some symptoms of osteoarthritis or lesions within the tissue. [32,33,41]. Because the chondrocytes are immobilized within the ECM, they may not be able to produce new matrix components at the defect site[37,41].

A variety of approaches to long term cartilage repair have been and are being explored in an effort to mimic the mechanical functions of cartilage tissue, improve mobility and quality of life. Treatments like physical therapy, bracing, and weight loss have been effective in relieving some symptoms associated with cartilage defects without surgery. While these methods are sometimes effective in providing temporary relief, they do not repair or regenerate damaged cartilage tissue[35]. Microfracture, a surgical treatment involving removal of damaged cartilage around a defect and drilling holes in the subchondral bone, results in some success by reducing shear stresses in the defective tissue, reducing pain, and allowing mesenchymal stem cells to migrate into the defect site[35,36,42]. This treatment must be followed up with some type of physical therapy regimen to introduce the mechanical forces needed to heal the site. Unfortunately, while this procedure can lead to chondrogenesis, it often results in fibrocartilage formation.[36]

Today, a number of approaches to cartilage repair focus on regenerating functional cartilage tissue, instead of temporarily supplementing defects. Such approaches include implantation of donated or engineered cartilage in the form of an allograft.[35,41] These allografts are generally successful in that the material will have similar mechanical properties of native cartilage, thus meeting many of the same functions. While this treatment can be successful in some cases, it is still limited in the size of the defect being treated, potential of an immune response, and the availability of donor tissue.[35,41] To avoid immune response, injection of autologous chondrocytes obtained from the patient can also be performed in a procedure known as autologous chondrocyte implantation. Chondrocytes are removed from the tissue, expanded in culture, and implanted back into the defect site. The chondrocytes then begin the process of producing cartilage matrix within the defect. While this treatment has had some success, there are issues with donor site morbidity, tissue scarcity, and poor long term function [35,36,41].

2.3 Stem Cells

One approach currently being developed to address this issue of replicating the natural process of chondrogenesis is utilizing multipotent stem cells. Stem cells are present throughout the body, and possess the ability to differentiate along a number of different lineages[5,6,43-50,92]. Current areas of study include analyses of soluble chondrogenic induction factors, 3D cultures, and mechanical conditions to successfully direct stem cell lineage specification. Early investigations, largely focusing on hMSC, have shown these cells demonstrate the ability to differentiate down a multitude of pathways in response to appropriate chemical [51] and physical [1,52-56] environments. Studies have included evaluation of critical growth factors, chemical supplements, and mechanical forces to induce MSC chondrogenesis[57,58]. While MSC hold great potential for functional cartilage tissue engineering, abundance and accessibility of MSC are limited. As a result, adipose derived stem cells ASC have gained great interest due to their similar multipotent differentiation ability to MSC and their relative abundance in donated tissue.[5,6, 15,48,52,59-70]. Functional tissue engineering and regenerative medicine studies with MSC and other cell types are now being explored with ASC to determine how best to utilize these cells for tissue repair and/or regeneration [71,5,6].While ASC and MSC are similar, there are distinct differences. Some studies have shown that ASC have less chondrogenic potential than hMSC, suggesting they may require greater treatments of growth factors and mechanical loading in order to produce similar results.[1,8-10,24,72-77]

2.4 Cartilage extracellular matrix.

The composition of articular cartilage varies with the position within the tissue, and is closely related to the mechanical function. Cartilage is stratified into five distinct layers transitioning into the subchondral bone: the superficial zone, intermediate or middle zone, the deep zone, tide mark, zone of calcified cartilage. The superficial zone is mostly acellular and made up of a network of type II collagen fibrils parallel to the surface of the joint. The intermediate and deep zones both include collagen II fibers of larger diameters, sulfated glycosaminoglycans, proteoglycans, and a

sparse community of chondrocytes. The large collagen fibrils of the deep zone run perpendicular to the joint surface, through the tidemark into the zone of calcified cartilage. The tidemark is the barrier between the more gelatinous cartilage tissue and the solidified tissue found in the zone of calcified cartilage, which anchors the tissue onto the subchondral bone.[59,78-80,40]

Many of the genetic markers associated with chondrocytes in the different layers of articular cartilage relate to the production of key proteins and proteoglycans embedded within the extracellular matrix. The most prevalent protein within the cartilage ECM is collagen II [59]. Collagen II forms into a network of fibrils where many of the other proteoglycans are embedded. This collagen network provides cartilage much of its functional tensile strength. The genetic marker most closely related to collagen II is Col2a1, which is expressed at all layers of articular cartilage [40]. Another critical constituent of the cartilage extracellular matrix is the glycosaminoglycan, hyaluronan, or hyaluronic acid [9,81,59]. The large chain like molecule is found in the intermediate and radial zones of articular cartilage as well as within the viscous synovial fluid which provides both lubrication and nutrients to articular cartilage. This molecule is known for its negatively charged side groups which contribute to the hydrophobicity of the molecule, and limit chain interaction due to electrostatic repulsion. While cartilage does contain a significant amount of water, the hydrophobic properties of cartilage keep it from becoming completely saturated, which maintains many of its mechanical properties.[9,81,59] Many of the proteoglycans associated with cartilage are attached to a hyaluronic acid backbone via small linking proteins. Aggrecan is the dominant proteoglycan in cartilage. Its configuration includes a three globule proteins section with two attached glycosaminoglycan chains, keratin sulfate, and chondroitin sulfate. The combination of proteoglycans and hyaluronic acid create the hydrogel consistency which provides the compressive strength of cartilage.[59]

2.5 Mechanical requirements of cartilage

The mechanical properties of the individual elements of cartilage are all necessary for overall function of the material. As the mechanical buffer between joints, cartilage has a variety of forces acting on it at varying magnitudes over varying durations. A common load associated with movement is unconfined compression, which affects the zonal layers of articular cartilage differently [138,83,80]. For example, standing, walking, and jumping all exert compressive forces on the cartilage of the hip joint ranging from 5 to 15 MPa [80]. The cartilage must be able to withstand static, dynamic, and cyclical loading without failure over a lifetime. Global compressive loads translate throughout the tissue, creating areas of local hydrostatic pressure within the tissue, fluid shear stresses as the fluid flows in and out in response to physiological loads, and shear strain at the cartilage interface with underlying bone. While cartilage is a low friction surface, joint loading creates tensile strains at the surface. Not only are the extracellular matrix constituents of cartilage specifically arranged and designed for these key mechanical roles, studies have shown that regular mechanical loading is a key precursor to matrix development and sustainment[138]. Natural loading has been shown to positively affect cartilage function and formation in vivo, and has also been shown to improve chondrocyte viability, gene expression, and proteoglycan formation in vitro, while excessive loading has been shown to lead to degradation of the ECM[38]. These studies demonstrating the response of cartilage to mechanical loading are critical to understanding both how defects propagate throughout the tissue, as well as provide key insight in cartilage tissue engineering.

2.6 Factors Affecting Chondrogenesis in vitro

Growth factors

Similar to natural chondrogenesis, the chemical environment plays a key role in chondrogenesis in vitro, particularly with various mesenchymal progenitor cells. Certain medium supplements have become somewhat standard for use in cartilage tissue engineering, including

Dulbecco's modified Eagle's medium (DMEM) , ITS (or ITS+), and various forms of ascorbate. Further, the presence of soluble growth factor has proven successful in chemically inducing chondrogenesis. Key Factors are those from the TGFβ super family, specifically, TGF β1, TGF β3 and BMP6. Our lab has previously reviewed these growth factors and their impact on chondrogenic differentiation of hMSC and hASC[84-93].

Shape

A major consideration in cartilage tissue engineering is the necessity of a three-dimensional (3D) culture environment. Studies have shown that stem cells cultured in 2D have diminished chondrogenic capabilities compared to 3D systems. Early studies have shown that cells with a spherical conformation produce collagen II, versus the collagen I produced by flattened fibroblastic cells[94,95] In addition to mimicking the environment of embryonic cartilage development, this shape also effects mechanotransduction of various types of loads, which in turn signals various processes critical to cartilage tissue development. Often, 3D culture is achieved by pelleting cells into a micromass. Other systems use various types of scaffolds, microspheres or hydrogels with varying degrees of success.

A number of key characteristics have impacted the success of each of these culture substrates, including their composition, stiffness, porosity, and fiber size. Biocompatible polymers like PLA and PGA have been used to create woven and nonwoven scaffolds able to biodegrade as the tissue is engineered[15,73,96-103]. Other scaffolds composed of decellularized cartilage have been shown to enhance chondrogenic differentiation due to the presence of certain ECM molecules[104-106]. Hydrogels mimic the natural composition of cartilage due to the high fluid content and gel consistency; however hydrogels are not always suitable for long term culture of cells without some cell adhesion ligands included in the formulation[15,107-121]. Microcarriers have also been an effective

method of maintaining the spherical shape of cells[122-126]. In each of these cases, the stiffness has been shown to be a key factor in differentiation, in that substrates of various stiffness have been shown to induce differentiation down specific lineages[127-130].

The substrate used to culture cells for chondrogenic differentiation greatly affects the way the cells receive key signals. In addition to maintaining the spherical shape of the cell, the 3D culture effects the way in which the cells interact with each other. Studies have shown the importance of high cell seeding density in chondrogenic differentiation of both MSC and ASC, suggesting that cell-cell signaling plays a key role in the process of chondrogenesis[12,131,135]. Further, the presence of certain molecules, like hyaluronan, enhances differentiation, suggesting that the cell's interaction with the ECM is another key aspect of the chondrogenesis process[106,118,132,133].

2.7 Mechanical loading

In addition to the chemical environment, and the physical culture conditions, mechanical loading has been shown to induce chondrogenesis, and depending on the type, amplitude, and frequency, can lead to a more mechanically functional engineered tissue. Table 1 highlights a number of studies using different forms of mechanical loading with various cell types and different constructs for cartilage tissue engineering. While the results of each study are different and show a variety of responses, some themes are prevalent. Other results show the importance of mechanical loading in conjunction with the proper chemical environment, and the importance of timing in the application of the force.

Author	Load	Cell type/ Medium/ Construct	Magnitude/ Frequency/ Duration
Blain EJ, (137)	Compressive Loading	Articular Cartilage/	.5 Mpa/ 1 Hz/ 3hr
Li J, (188)	Compressive Loading	ASC/ Chitosan-Gelatin Scaffolds	5%, 1hz, 4 days
Bahuleyan B.,(16)	Compressive Loading	Rabbit MSC/ TGF B1	10%/ 1 Hz
Elder SH, (186)	Compressive Loading	Chick limb-bud/Agarose	.03-.33Hz/12mn-1hr
Huang CY, (139)	Compressive Loading	Rabbit MSC/	.1,1,10 MPa/1Hz
Kisiday JD, (138)	Compressive Loading	MSC/ CDM w/wo TGFβ / Agarose hydrogel	2kPa/ .3Hz/ 6hr or 12 hr per day
Takahashi I, (134)	Compressive Loading	Mouse Embryonic Limb-bud cells/ Collagen gel	.5, 1 g/cm^{3}static
Li Z Y,(140)	Compressive Loading/ shear stress	MSC/ Fibrin Polyurethane Composite	15, 20, 30%/.1,1Hz/ 1hr per day/ 7 days
Mauck RL, (136)	Confined Compression/ tensile	Chondrocytes/ Agarose & Alginate Hydrogels	10%, 1Hz
Pelaez D, (141)	Cyclic compression	MSC/ Fibrin Gel	10%/1Hz/ 4hr/day 33 days
Mizuno S, (144	Cyclic Hydrostatic Pressure	bovine Chondrocytes/ collagen sponges	2.8Mpa/ .015 Hz/ 33ml/min
Ogawa R,(161)	Cyclic Hydrostatic Pressure	ASC/CDM Collagen Scaffold	0-.5 MPa/ .5Hz
Li Z, (189)	Dynamic Compressive Loading	MSC/ fibrin-polyurethane hybrid system	10–20%/ 1hz/ 1 hr a day over
Thorpe SD, (142)	Dynamic Compressive Loading	MSC/ CDM/	10%/ 5Hz/ 1hr, 5 days
Tagil M, (174)	Dynamic	MSC/CDM	2 MPA/.17 Hz/2x per

	Compressive Loading		day/20 cycles
Huang AH, (143)	Dynamic Unconfined Compression	Bovine Bone Marrow Stem Cells	10% dynamic /2%static / 1hz, 4hr/day
Toyoda T,(145)	Hydrostatic Pressure	Bovine Chondrocytes/	5 Mpa/ 1Hz/ 4hr
Angele p, (150)	Hydrostatic Pressure	MSC/ Serum Free medium	10 MPa/ 1Hz
Angele P, (151)	Hydrostatic Pressure	MSC/CDM	5.03 MPa/1Hz/ 14-28 days
Finger AR,(152)	Hydrostatic Pressure	MSC/Agarose w/o TGFB	7.5 MPa/1HZ
Finger AR, (152)	Hydrostatic Pressure	MSC/TGFB3	.1,1,10 MPa/1Hz
Hall AC,(146)	Hydrostatic Pressure	Bovine Chondrocytes/	2.5-50 MPa
Ikenoue T,(147)	Hydrostatic Pressure	Articular Chondrocytes	1, 5, 10 Mpa/ 1Hz/ 4hr
Miyanishi K,(149)	Hydrostatic Pressure	MSC/ CDM	10 MPa/1Hz/ 3, 7, 14 Days
Mukherjee N,(193)	Hydrostatic Pressure	periosteol graft	13, 54, 103 kPa, 5, 4, 24hrs
Reza AT, (195)	Hydrostatic Pressure	Chondrocytes (OA,IA,NP)/PGA-PLLA Scaffolds	5MPa, .5 Hz, 4hr,12 days
Lee, Mel, (148)	IHP/Shear Stress	OA Chondracytes/ serum free medium	1.6Pa/ 200RPM/2hr/10Mpa/1HZ/ 4hr/day 1,2,4 days
Carver SE,(173)	Hydrostatic pressure, fluid shear, and mixing	Articular Chondrocytes/ PGA scaffold	50 RPM/ 13.5 MPa/ 3.44 MPa
Wimmer M, (200)	Shear stress	Chondrocytes/PU Scaffold	.28,2.8, 28 mm/s, 1hz,1hr/ 2x Per day, 5 days
Kupcsik L, (185)	Shear stress/ dynamic compression	MSC/ Fibrin PU scaffold	25o/ 1 Hz / 10-20%/ 1 hr/ 14 days
Diederichs S, (176)	Shear Stress/Tensile strain	Embryonic Rat Limb-bud/Silicon	5%/1Hz/ 15 min-8 Hr/ 2x per day
Kuo CK, (170)	Tensile strain	MSC/ Collagen gel	1%/ 1Hz/ 30min per day
Connelly JT,(175)	Tensile strain	MSC/CDM/ Fibrin Hydrogel	10%/ 1 Hz/ 3hrs
Tanaka T,(159)	Tensile strain	Rat Chondrocytes	7, 12%/ .5HZ

Table 1. Mechanical loading for Chondrogenesis

Compression

Compressive forces have been the most effective and studied force applied to induce chondrogenesis. Compressive forces are experienced throughout the process of endochondral ossification, as well as normal joint function and have been shown to increase aggrecan synthesis and matrix development in vivo[38]. In studies using embryonic limb bud progenitor cells, dynamic compressive loading was shown to be more effective in driving chondrogenesis than static loading as evidenced by increased cartilage nodules. Static compressive loading resulted in a 2-3 fold increase in collagen II, aggrecan and SOX9 expression over unloaded samples[134,135]. Mauck[136] demonstrated that dynamic loading of a chondrocyte seeded agarose disc not only increased the hydroxyproline and sulfated GAG contents, but also increased the equilibrium aggregate modulus 6 fold over free-swelling controls, and 20 fold over the 28 day period. These findings demonstrate that compressive loading not only improves matrix production, but also helps develop mechanically functional tissue. Blain et al[137] demonstrated though that cyclic compression of bovine cartilage explants resulted in elevated expression of matrix metalloproteinases, MMP, which are prevalent in matrix remodeling. This demonstrates the effectiveness of compressive loading on the metabolic activities of cartilage tissues, but also shows a mechanism in which excessive loading can lead to tissue degradation [137].

In contrast with these studies, which focused on cartilage tissue, chondrocytes, and cartilage progenitor cells, compressive loading on MSC and ASC must control differentiation as well as

stimulate matrix production. A by Kisiday et al [138] demonstrated that a compressive loading protocol necessary to induce chondrogenic differentiation in MSC may not be best for new tissue accumulation. In the absence of soluble chondrogenic growth factor TGF-β, dynamically compressed constructs demonstrated increased levels of sulfated GAGs, and hydroxyproline over unloaded controls, similar to the results seen with other cell sources. However, the GAG content of dynamically loaded constructs cultured in the absence of TGF-β was significantly lower than that of unloaded constructs loaded in the presence of TGF-β. Further, when both conditions were cultured with TGF-β, dynamically compressed samples showed decreased GAG content compared to unloaded controls [138]. Huang et al [139] conducted a similar experiment suggesting that when the loading is done over a longer period of time, compressive loading is as effective in chondrogenesis as both TGF-β, and TGF-β with compressive loading, suggesting that the dynamic compressive loading led to the synthesis of TGF-β1 by the MSC in culture, which in turn leads to chondrogenic differentiation [139]. Subsequent studies further demonstrated how compressive loading leads to TGF-β1 and β3 synthesis in MSC, and that greater amplitudes and frequencies of loading, over a longer culture period, generally lead to increased chondrogenesis [140,141]. Some studies have shown limits of compressive loading on inducing chondrogenesis, including the time point to being a loading regimen, or the mechanical properties of the construct. Unless the construct is able to properly conduct the compressive load, chondrogenesis can be inhibited.[142] Huang et al[143] showed loading conducted prior to chondrogenic differentiation of MSC led to less functional matrix material, even though expression of chondrogenic markers was increased. In contrast, when loading occurred after chondrogenesis, and TGF-β3 levels were maintained, mechanical properties were improved.[143] These studies demonstrate the relationship between the TGFβ super family, compressive loading, and cartilage tissue engineering.

Hydrostatic Pressure

In studies conducted with articular chondrocytes, hydrostatic fluid pressure, HFP, has been shown to stimulate the production of cartilage ECM greater than ambient pressures [144-147]. Mizuno et al [144] demonstrated how articular chondrocytes, in an appropriate 3D construct experienced increased sulfated GAG content under both cyclic and constant hydrostatic pressure. Other studies demonstrated the effects of hydrostatic pressure on cell shape, cell-matrix interactions, and cell surface proteins, demonstrating how each play a role in tissue metabolism. One such study by Lee et al [148] demonstrated that cyclic hydrostatic pressure inhibited the catabolic effects of inflammatory cytokines [148]. As with compression, the magnitude, and duration of treatment, along with the mechanical properties of the cell construct greatly impact the effect of hydrostatic loading. However physiological levels of hydrostatic pressure in articular cartilage (in the range of 2-5 MPa) have been effective in increasing matrix production in articular chondrocytes [145-149].

Similar results of increased matrix production in response to hydrostatic pressure were seen in studies using MSC [150]. Angele et al [151] demonstrated an increase of proteoglycans due yo CHP loading on cell aggregates in chondrogenic medium, where as our lab has previously demonstrated that CHP was effective in increase early chondrogenic differentiation markers in MSC constructs cultured without known chondrogenic growth factors such as TGF-$\beta 3$ [152,106]. These studies are important in that they demonstrate that CHP is effective of increasing matrix production in stem cells being directed down a chondrogenic pathway, as well as inducing chondrogenic differentiation in stem cells.

Shear stress

Shear stresses have had various dose and time dependent effects on cartilage tissue engineering and chondrogenic differentiation. Some studies have shown the use of shear strain in the range of 1-3% leading to increases in both the amount and length of proteoglycans and matrix

proteins in cultured articular chondrocytes [153, 154]. Additionally, Smith et al [154] demonstrated that chondrocytes produce MMP inhibitors in response to fluid shear, demonstrating that fluid shear plays a role in normal cartilage tissue metabolism. Smith later demonstrated that shear stresses may work in concert with other mechanical forces to maintain homeostasis [155]. Where compression and hydrostatic pressure resulted in increased production of aggrecan and type II collagen, fluid shear resulted in lower level, of both aggrecan and type II collagen, and an increase of nitric oxide. This study and others also suggest that fluid shear can result in apoptotic conditions in chondrocytes, furthering the damage associated with osteoarthritis. [154]. Bushman et al[156] demonstrated that fluid shear resulting from dynamic compression led to increased aggrecan production, suggesting a need for a combination of mechanical loads in order to maximize the effectiveness in each with regards to cartilage tissue engineering. This is consistent with Mow et al [83] description of the different load types experienced in various regions of cartilage tissue in response to natural motion. Considering these results, fluid shear should be applied to engineered cartilage tissues as a mediator of natural cartilage metabolism, in concert with other types of mechanical loading.

Tensile strain

Tensile strain is normally associated with bone tissue engineering, because this loading type generally leads to elongated and organized cells, which can lead to fibrocartilage tissue engineering.[157,158] Tanaka et al [159] demonstrated that applying cyclic tensile strain to chondrocytes resulted in the secretion of PTHrP during the proliferative and matrix producing stages. This study went on to suggest that the presence of PTHrP may improve chondrocyte proliferation and matrix production, which in turn would improve cartilage tissue. Another study by Onodera et al [160] demonstrated that stepwise mechanical stretching resulted in the inhibition of chondrogenesis of rat limb-bud cells cultured in micromass. This inhibition was recovered however when cell matrix

adhesion was inhibited, suggesting the negative effects of tensile strain are based on the interaction between cells and the matrix.

ASC

Although a number of studies have examined the impact of various mechanical loads on cartilage tissue engineering, as well as mechanical loads on ASC (Table 2) few studies have focused on mechanical loading of ASC in cartilage tissue engineering. Cyclic hydrostatic pressure has been shown to enhance chondrogenic differentiation in ASC, in the presence of soluble chondrogenic growth factors, TGF-β3 [161]. In our lab we have previously shown CHP to be effective in inducing chondrogenic differentiation in ASC without the use of chondrogenic differentiation medium or soluble growth factors [107]. Both studies demonstrated the importance of an appropriate construct for long term cell viability and proliferation. Because of the limited work studying the effects of mechanical loading on cartilage tissue engineering with ASC, we must extrapolate expected results based on other studies utilizing mechanical loading and ASC. Compression was shown to improve cartilage tissue development because of an increase in growth factors like TGF-β3. A number of studies have compared the chondrogenic potential of ASC compared to MSC, and have concluded that MSC have greater chondrogenic potential than ASC under identical culture conditions including the presence of TGF-β3 ([11,15,51]). This suggests that ASC may need a greater magnitudes and durations of compressive loading, or TGF-β3 in greater doses during mechanical loading. Table 2 lists a number of studies observing the effects of mechanical loading on ASC, demonstrating the response of this cell type under different conditions.

Author	Load	Frequency/Amplitude	Tissue
Li J, (188)	Compression	5%, 1hz, 4 days	Cartilage
Ogawa R, (161)	Cyclic Hydrostatic Pressure	0-.5 MPa/ .5Hz	Bone
Puetzer J (107)	Cyclic Hydrostatic Pressure	7.5 Mpa,1Hz, 4h/d, 21 days	Cartilage
Knippenberg M,(184)	Fluid shear	8.4 Pa/s / 5, 10, 15, 30, 60 min	Bone
Bassaneze V, (171)	Shear stress	10 dynes/cm^2, 96hr	Vascular
Fischer LJ, (178)	Shear stress	12 dynes, 8 days	Endothelial
McIlhenny SE, (192)	Shear stress	ramped at 1.5dynes/s	Vascular
Hanson AD, (179)	Tensile strain	10%/ 1Hz/ 4hr per day	Bone
Bayati V, (172)	Tensile strain	10%, 1 Hz, 24 hr	Muscle
Diederichs S,(176)	Tensile strain	5%, 1hz, .25-8 hr	Bone
Huang S-C,(180)	Tensile strain	10%.5hz, 48hr	Proliferation
Lee W,(187)	Tensile strain	10%, 1hz, 7 days	Inhibition
Wall ME, (199)	Tensile strain	10%, 1hz, 4hr, 14 days	Bone
Pre D, (194)	Vibration	30hz, 45 min, 28 days	Bone

Table 2. Mechanical Loading and ASC

2.8 Mechanotransduction

The observation of various mechanical loads by the cell, while not fully understood, is attributed to the presence of various mechanotransducers which translate the various mechanical loads into a chemical signal to the cell. The translation of extracellular load to a signal within the cell can be directed by force-sensitive ion channels, like stretch-activated ion channels, or interactions between the cell membrane and the ECM. [130,162]Some mechanical forces are transduced by membrane bound proteins interacting with the actin cytoskeleton or extracellular matrix, initiating signally pathways within the cell such as the Wnt, RhoA, or MAL signaling pathway[59,163]. Recently,

the primary cilium has been studied as a cellular mechanosensor directing stem cell differentiation. [164] This organelle has been shown to be a key mechanosensor in bone and cartilage tissue, sensing flow, compression, and hydrostatic pressure, directing metabolic processes as well as differentiation[165-169]. The cilia signal the cell to mechanical forces by directing an increase of intracellular Ca^{2+} levels through the permeable ion channel Polycystin 2[166]. Roberts et al his demonstrates that intracellular Ca^{2+} concentrations spiked in cells undergoing compressive loading [204]. This spike was generally observed by 200 s after the load was applied followed by a slow return to homeostasis [204]. This demonstrates the important role of Ca^{2+} in mechanotransduction.

2.9 Conclusions

The nature and function of cartilage tissue translates regular movement into various forms of mechanical loads throughout the region, each playing an important role in the homeostasis of the tissue. As we improve our methods of cartilage tissue engineering, biomimetic mechanical loading has been shown to be critical to engineering mechanically functional tissue. Additionally, the compressive forces and hydrostatic pressure present during embryonic endochondral ossification seem equally important in directing stem cell differentiation. Further, other factors such as the chemical environment, mechanical properties of the cell construct, and the biological state of the cells are integral in determining the appropriate magnitude, duration, and type of mechanical load needed to optimize cartilage tissue engineering. It is clear however that more research needs to be done to better understand the mechanisms by which these loading modalities impact differentiation. Cartilage tissue engineering must leverage both chemical and mechanical factors in order to maximize the effectiveness of hASC.

Chapter 3:

Elevated Extracellular Calcium Inhibits Chondrogenic Differentiation of Human Adipose-Derived Stem Cells

The previous chapter discussed the role of mechanical loading in hASC differentiation. The mechanism by which a number of mechanical forces direct cell activity is through mechanosensing ion channels and ion permeable membranes. During mechanical loading, cells experience a rapid increase in intracellular Ca^{2+} concentration, translating the mechanical load to a chemical signal. This demonstrates a function of Ca^{2+} in cell signaling which may be leveraged without the use of mechanical loading. Previous studies have shown that elevating extracellular Ca^{2+} concentration to 8mM is effective in increasing hASC mediated calcium accretion and osteogenic differentiation. Based on the role of Ca^{2+} in cell signaling, as well as the impact of elevated Ca^{2+} on hASC differentiation, this study aims to observe the impact of elevated Ca^{2+} on chondrogenic differentiation of hASC.

3.1 Introduction

Calcium is a critical ion in cell biology, playing a key role in a number of cell functions necessary for cell homeostasis. Important cell functions are often directed by the transport of calcium ions across the cell membrane, into the cytoplasm from intracellular stores, or the allosteric binding of calcium to a protein[1, 2]. Calcium can act as a second messenger translating mechanical load to a chemical signal directing cell activity, as with certain stretch activated mechanostransducers[2.3]. It can further act as a first messenger, binding with proteins outside of cell membranes, causing a cascade of signals within the cell[4].

Calcium ions can also affect stem cell proliferation and differentiation. Our lab has previously observed the effects of elevating Ca^{2+} levels in complete growth and osteogenic differentiation media on hASC, comparing mineralization and proliferation. That study demonstrated that elevation of extracellular Ca^{2+} in complete growth medium to a concentration of 8mM was effective in increasing human adipose derived stem cell (hASC) production of mineralized matrix, an indicator of osteogenic differentiation[5]. The use of elevated Ca^{2+} to direct hASC osteogenic lineage specification is a clear advantage in bone tissue engineering, however, the impact of elevated Ca^{2+} has not been widely studied in cartilage tissue engineering.

Articular cartilage is found at the end of long bones with a primary function of supporting locomotion by bearing the repetitive mechanical loads associated with movement. The tissue is avascular with limited regenerative capability, which limits its ability to integrate engineered repair tissue. Bone, in contrast, is highly vascular with regular remodeling processes. Future cartilage engineering endeavors may leverage the regenerative properties of nearby bone to ensure implants are functionally integrated into the joint. In order to accomplish this, the impact of osteogenic factors on chondrogenesis must be better understood. This aim of this study was to determine the effect of elevated Ca^{2+} on chondrogenic differentiation of hASC.

3.2 Materials and Methods

hASC Isolation and Culture

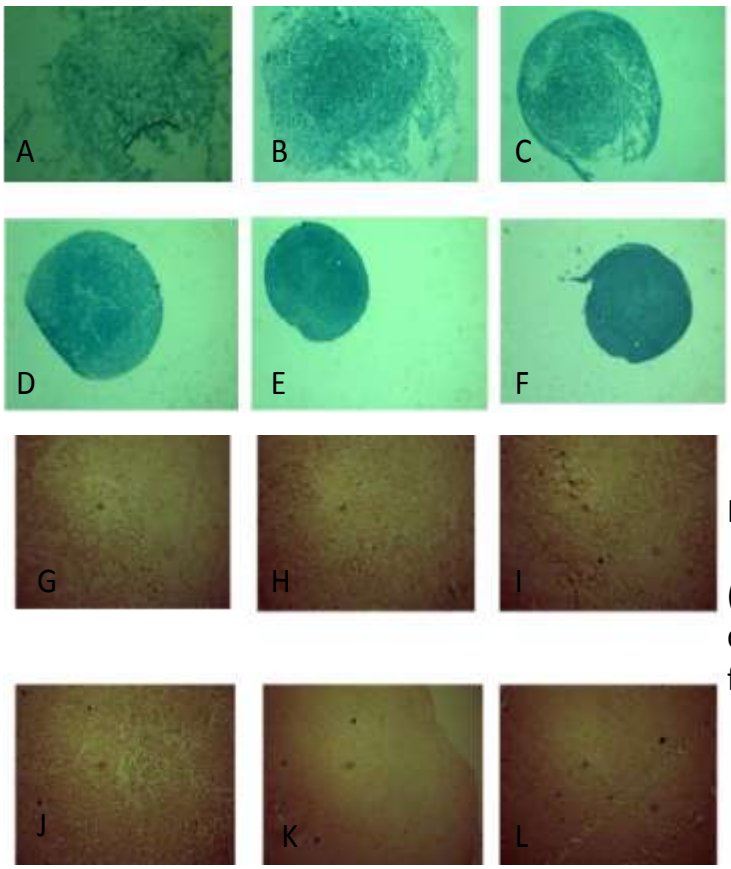
Excess adipose tissue was obtained from five donors (24 to 37 year old females) in accordance with an approved IRB protocol at UNC Chapel Hill (IRB 04-1622). Human ASC were isolated from the tissue using a method described by Zuk et al[6] as previously described by our lab[7, 8]. At passage 2, 100K cells of each cell line were seeded in a single T-75 flask, in complete growth medium (CGM) comprised of alpha-modified minimal essential medium (α-MEM with L-glutamine) (Invitrogen) supplemented with

10% fetal bovine serum (FBS) (Premium Select, Atlanta Biologicals, Lawrenceville, GA), 200 mM L-glutamine, and 100 I.U. penicillin/ 100 µg streptomycin per ml (Mediatech, Inc.) The cells were allowed to proliferate at 37°C in 5% carbon dioxide until reaching 70% confluency, and then trypsinized. The amassed cells were then characterized for osteogenic and adipogenic potential, ensuring the amassed cells differentiated representative of an average of the five cell lines.

Chondrogenic Characterization/Medium Formulation

Suspensions of 250K hASC were centrifuged in 1 ml of media into micromassed pellets. Pellets were cultured in one of six chondrogenic differentiation medium formulations. Formula 1 was comprised of Dulbecco's modified Eagle's medium (Mediatech, 15-013-CV), 1% dexamethasone (Sigma Aldrich, D4902), 1% ITS+ (Sigma-aldrich, I2521), 1%Pen/Strep, and 1% ascorbic acid (Sigma, A4403). Formula 2 included all components of Formula 1 with the addition of 1% TGF-β1 (R&D Systems, 100-B) at a concentration of 10ng/ml. Formula 3 included all components of Formula 2 plus 1% bone morphogenetic protein 6 (BMP6, R&D Systems 507-BP) at a concentration of 10ng/ml. Formulas 4 through 6 were comprised of Formulas 1 through 3, respectively, but with the addition of 10% fetal bovine serum (FBS) added to Formulas 1 through 3. Pellets were cultured at 37°C in 5% carbon dioxide for 21 days, receiving conditioned media changes every 48 hours. Conditioned media changes remove only half of media within culture, in order to preserve growth factors secreted by cells. Ascorbic acid and chondrogenic growth factors, TGF-β1 and BMP6, were added to each medium formulation immediately prior to conditioned media changes. Cell pellets were then dehydrated, blocked in paraffin, and sectioned for histological analyses. Sections were stained with alcian blue (Sigma, A9186) for glycosaminoglycan (GAG) content, as well as immunohistochemical (IHC) staining for collagen type II. IHC was conducted with a Histostain-plus kit (Invitrogen, CAT 85-8943) utilizing primary antibodies for

collagen type II (DSHB, II-II6B). Based on IHC and histological results (Figure 1), Formula 6 was chosen for all further experiments.



Group	Components
Group 1	DMEM, ITS+, Pen/Strep, Dexamethasone, Ascorbic acid
Group 2	DMEM, ITS+, Pen/Strep, Dexamethasone, Ascorbic Acid, TGF-β1
Group 3	DMEM, ITS+, Pen/Strep, Dexamethasone, Ascorbic Acid, TGF-β1, BMP-6
Group 4	DMEM, FBS, ITS+, Pen/Strep, Dexamethasone, Ascorbic acid
Group 5	DMEM, FBS, ITS+, Pen/Strep, Dexamethasone, Ascorbic acid, TGF-β1
Group 6	DMEM, FBS, ITS+, Pen/Strep, Dexamethasone, Ascorbic acidTGF-β1, BMP-6

TGF - β1 : 10ng/ml BMP-6 : 10ng/ml

M

Figure 1. Alcian blue stain of pellets cultured in six different CDM formulas (A-F). IHC for collagen II in pellets cultured in six different CDM formulas (G-L). Media formulations (M)

Pellet culture

Human ASC pellets were made as previously described, and cultured in the selected chondrogenic differentiation medium at either basal levels of Ca^{2+} (1.8 mM) or 8mM Ca^{2+}. Ca^{2+}

concentration was elevated by dissolving $CaCl_2$ (Sigma, C7902) in the culture medium. Pellets were cultured in the same manner as previously described.

Histological Analysis

Pellets from each condition were collected on day 21 for histological analysis. Pellets were fixed in 10% formalin, dehydrated, and embedded in paraffin blocks. The paraffin blocks were sectioned and stained with Safranan O/Fastgreen FCF (Fisher Chemical) /Hematoxylin (Sigma) for cartilage tissue, alcian blue for sulfated GAG content, and alizarin red for calcium deposition. Immunohistochemistry was conducted using primary antibodies for collagen type II, as well as types I and X (Abcam, ab90395, Sigma, c7974). The sections were viewed at room temperature with a Leica DM LFSA microscope (Wetzlar, Germany) equipped with a 40x water immersion, high resolution camera (Hamamatsu City, Japan), and SimplePCI image capture and analysis software (Compix, Sewickley, PA).

Real-time reverse transcriptase polymerase chain reaction

Pellets from each condition were collected on days 0, 7, 14, and 21 of culture for quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Each construct was dissolved in RNA lysis buffer and total RNA was isolated using a Perfect RNA Eukaryotic Mini kit (Eppendorf, Westbury, NY). Complementary DNA was synthesized using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA) with oligo $(dt)_{20}$ primers. Primers and probes for human collagen II (Assay HS00264051 M1), aggrecan (Assay HS00153936 M1), Sox9 (Assay HS00165814 M1), Cartilage oligomeric matrix protein (COMP, Assay HS00164359 M1), Runx 2 (Assay HS00231692 M1), Alkaline Phosphatase (ALP, Assay HS01029144 M1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Assay HS99999905 M1) were purchased from Assays-on-demand (Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed using TaqMan PCR Master Mix (Applied Biosystems) in an

ABI Prism 7000 system (Applied Biosystems). Signals were normalized to GAPDH expression levels using the $-\Delta\Delta$CT method[9].

DNA Quantification

DNA quantification of pellets from each condition was conducted utilizing a picogreen dsDNA quantification assay (Invitrogen), and a modified protocol provided by Molecular Probes Protocols (MP07581). Samples were digested in a solution of Papain, phosphate buffered saline (PBS), L-Cysteine, and EDTA overnight at 65^{o}. Digested samples and prepared DNA standards were combined with a Picogreen dye solution in a 96 well microplate, and allowed to incubate at room temperature protected from light. Fluorescence of the samples and standards was obtained by a Tecan GENios with Magellan 5 software (Tecan, Zurich, Switzerland) exciting samples at 480nm and reading emissions at 530nm. Readings were corrected and converted to concentrations of DNA for each sample.

DMB Assay

Previously digested samples were analyzed for sulfated GAG content using a modified spectrophotometric DMB assay. DMB dye was prepared by combining 1,9-dimethlemethylene blue (Sigma-Aldrich, St. Louis, MO) with ethanol and sodium formate (Sigma-Aldrich, St. Louis, MO). Digested samples and prepared standards of chondroitin-4-sulfate were combined with DMB dye in a 96-well microplate. Optical density was measured at 595 nm using a Tecan GENios with Magellan 5 software (Tecan, Zurich, Switzerland). Corrected readings were converted to concentrations of sulfated GAGs.

Hydroxyproline assay

Quantification of hydroxyproline, a component of collagen type II, was conducted using a modified hydroxyproline assay (Stegman, Neidert). Samples previously digested in Papain solution, and hydroxyproline standard solutions were hydrolyzed with 12N HCL at 110^{o} C overnight. Samples and

standards were then dried for 72 hours, and reconstituted with an acetate-citrate buffer solution (sodium acetate trihydrate, citric acid monohydrate, acetic acid, and sodium hydroxide). Reconstituted samples and standards were centrifuged through activated charcoal and .4 micron filters. Filtered samples were then oxidized with a fresh chloramine-T reagent (.062 M, chloramine-T, propanol, acetate-citrate buffer) in a 96 well microplate, and agitated for 15 minutes at room temperature. Then, p-DMBA reagent (.94 M, p-dimethylaminobenzaldehyde, propanol, perchloric acid) was added to each well, and incubated at 37°C for thirty minutes, allowing chromophore development. Optical density at 550 nm was measured using a Tecan GENios with Magellan 5 software (Tecan, Zurich, Switzerland). Corrected readings were converted to concentrations of hydroxyproline.

3.3 Results

Histological Analyses

All samples showed distinct morphological differences between elevated 8mM Ca^{2+} experimental pellet sections, and sections of 1.8mM Ca^{2+} control pellets. The outer portions of pellets cultured in 8 mM Ca^{2+} appeared stratified and loose, whereas control pellets cultured in 1.8 mM Ca^{2+} were uniformly dense and compact. Alcian blue staining for GAGs was positive for both control and experimental pellets, though slightly greater in control 1.8 mM Ca^{2+} pellets (Fig 2). Alizarin red staining indicated significantly higher mineralization in the elevated 8 mM Ca^{2+} pellet, especially towards the center of the pellet section, relative to the control (Fig 3). Safranan O/Fastgreen FCF staining showed similar staining of GAG and collagen content for both experimental and control samples, however, this stain showed a difference in the distribution of cells within the pellet sections (Fig 3). Pellets cultured in 8 mM Ca^{2+} showed a sparse community of cells in the loose outer bands of the pellet, and a more uniform distribution of cells within the center. Control 1.8 mM Ca^{2+} pellets had a higher concentration of cells towards the outer portions of the pellet, and a sparse population of cells in the center of the pellet. Immunohistochemical analysis of the pellet sections for collagen type I was positive for both

experimental and control pellets, however, the location of positive staining within the pellets were slightly different. In the experimental pellet section, positive staining for collagen type I was more prevalent in the loose outer rings of the pellets, whereas positive staining within the control pellet took on a swirl pattern within the center of the pellet. Control pellets stained more positive for collagen type II than experimental pellet section. Positive staining was seen uniformly throughout the control pellet sections, but variable within the experimental pellet sections. Both 1.8 mM and 8 mM Ca^{2+} pellet sections stained similarly for collagen type X within the center of each section; however 8 mM Ca^{2+} pellets had greater collagen type X staining in the loose outer rings of the sections relative to the controls (Fig 4).

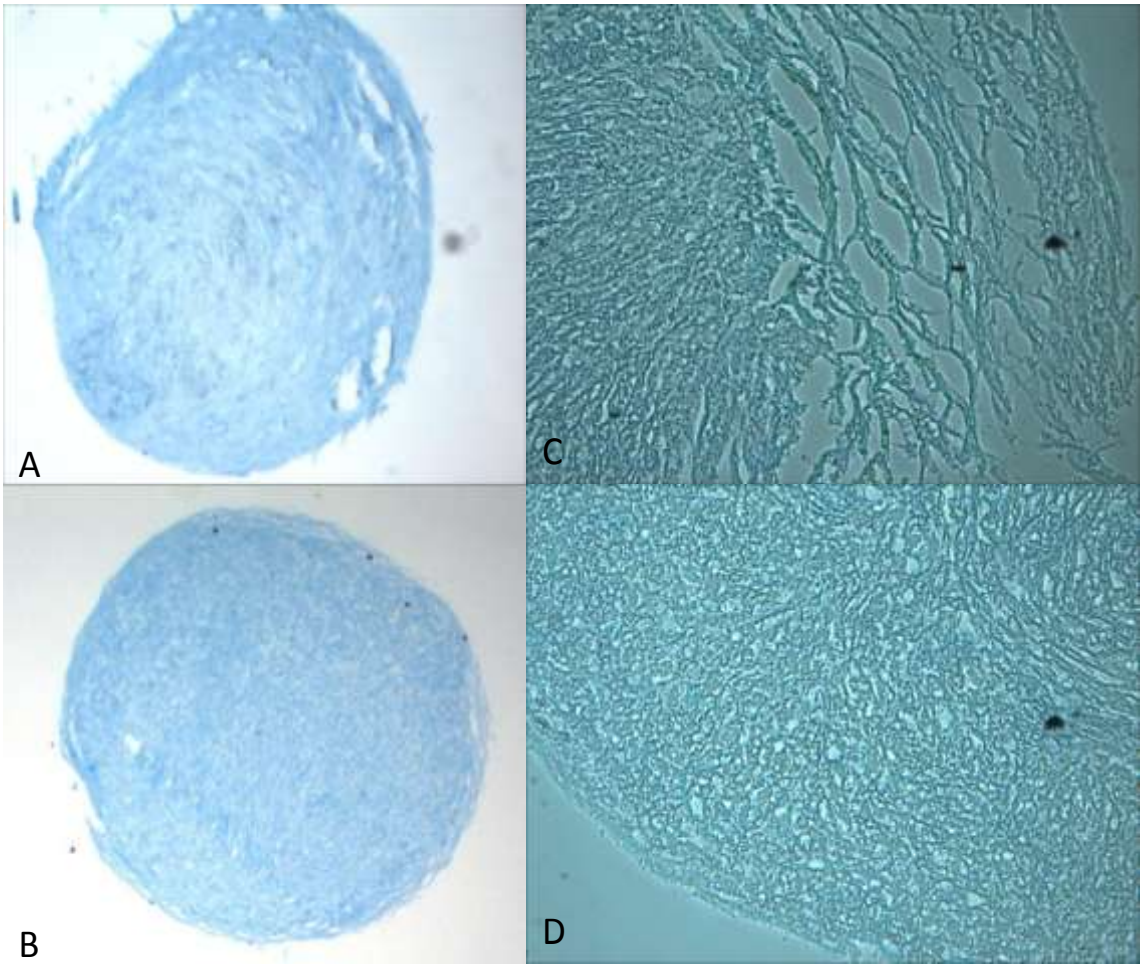


Figure 2. Alcian Blue staining of 8mM Ca^{2+} pellet sections (A,C) and control 1.8 mM Ca^{2+} pellet sections (B,D)

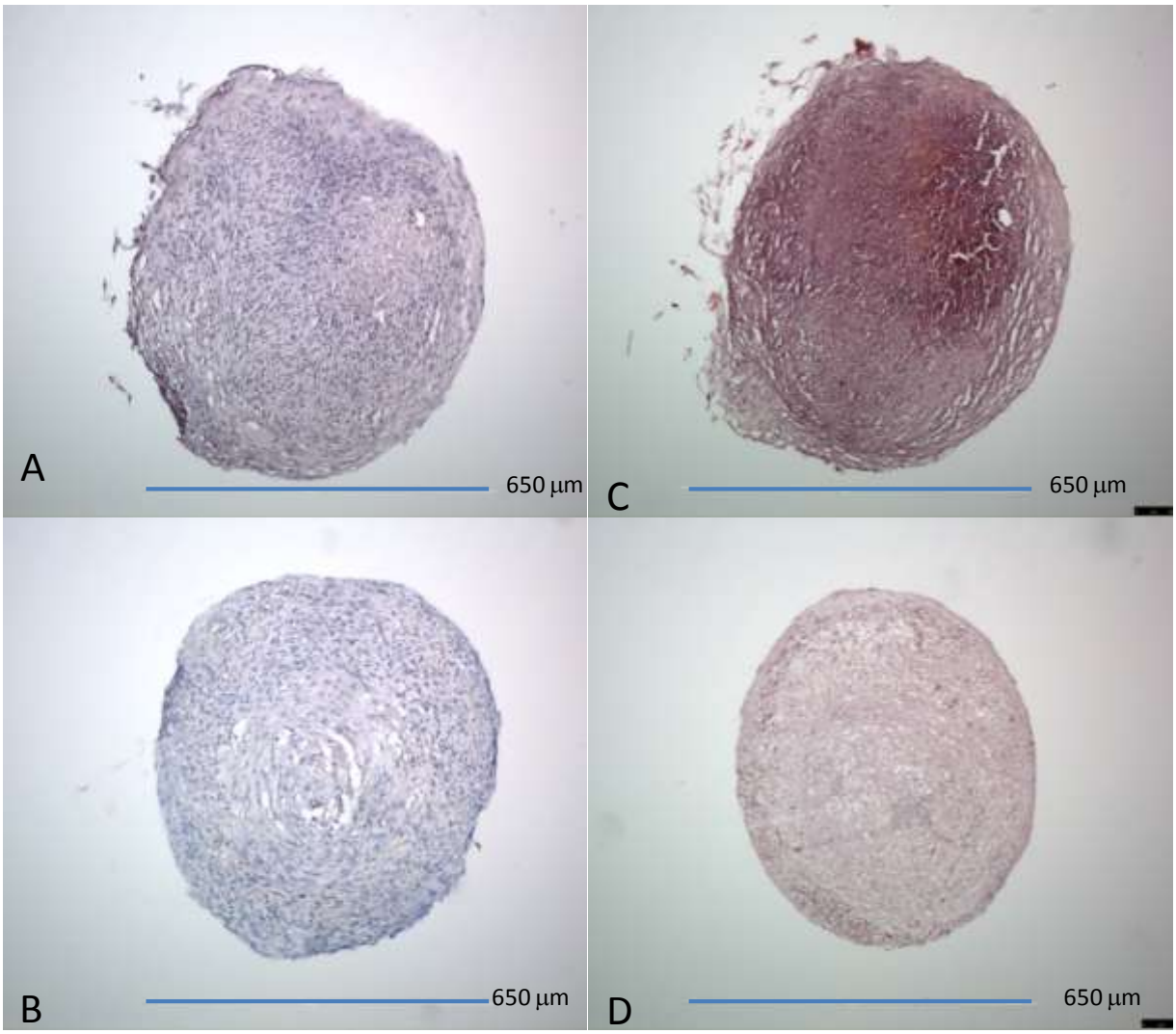


Figure 3. Safranan O/FCF Fastgreen/Hematoxylin and Alizarin red stains of 8mM Ca^{2+} Pellet sections (A,C) and control 1.8mM Ca^{2+} pellet sections (B,D)

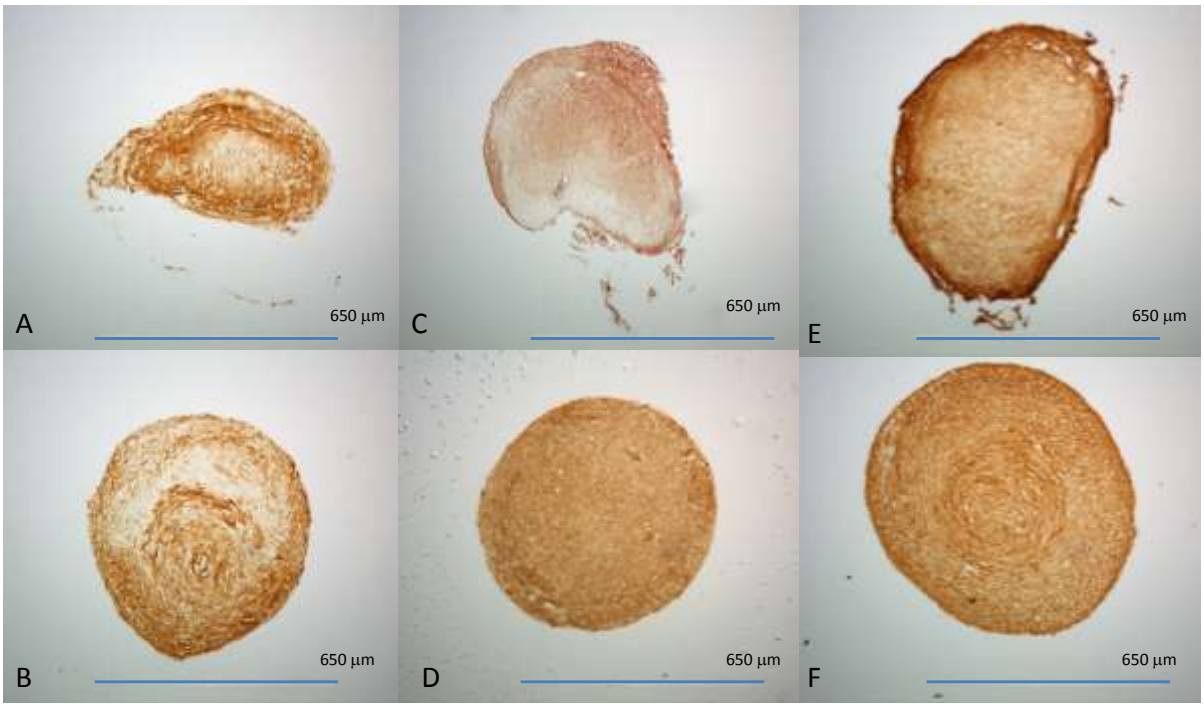


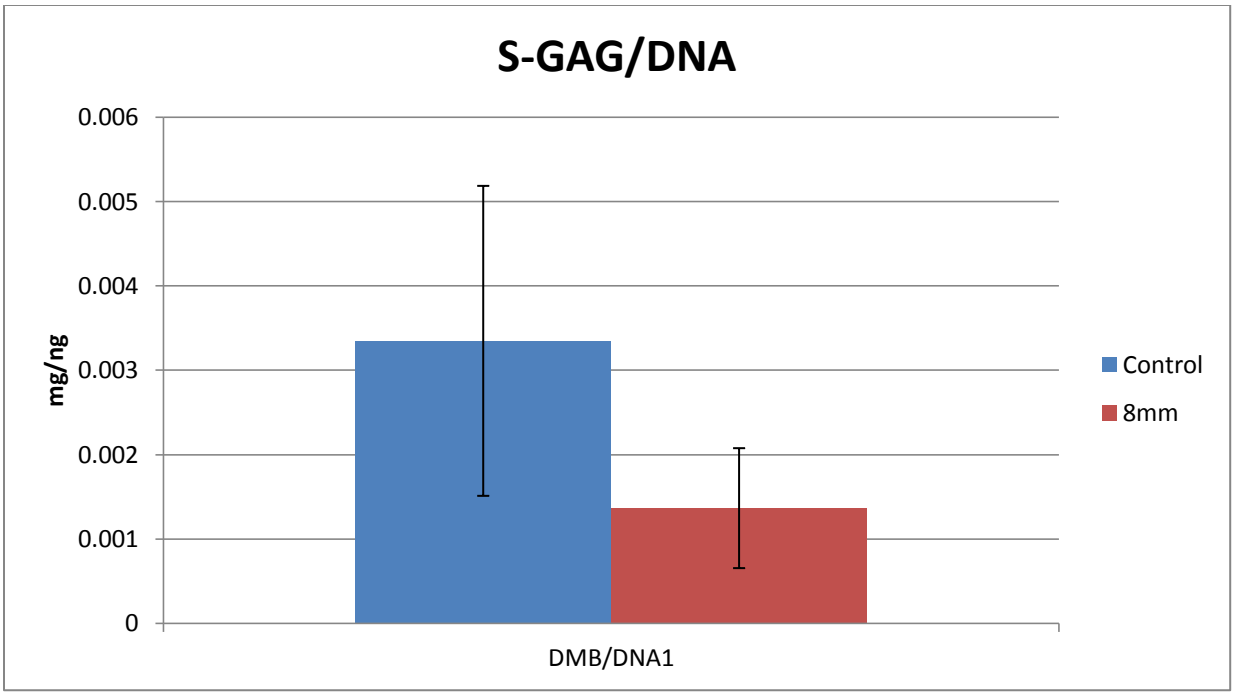
Figure 4. IHC of pellet sections for col I (A-8mM Ca^{2+}, B-control), col II (C-8mM Ca^{2+}, D-control), and col X (E-8mM Ca^{2+}, F-control)

Real time RT-PCR Analyses

No significant differences were found in mRNA expression between the 8 mM Ca^{2+} samples and 1.8 mM controls. Experimental pellets demonstrated diminished aggrecan mRNA expression at day 7 relative to controls; however this difference was not statistically significant.

Sulfated GAG and Hydroxyproline

Calculated values for both sulfated GAGs and hydroxyproline were normalized to DNA for both experimental and control pellets. The DMB assay indicated a decreased ratio of sGAGs/DNA in 8 mM Ca^{2+} pellets relative to 1.8 mM Ca^{2+} controls. The hydroxyproline/DNA ratio of the 8 mM Ca^{2+} pellet was also lower than the calculated values from the control (Fig 5)



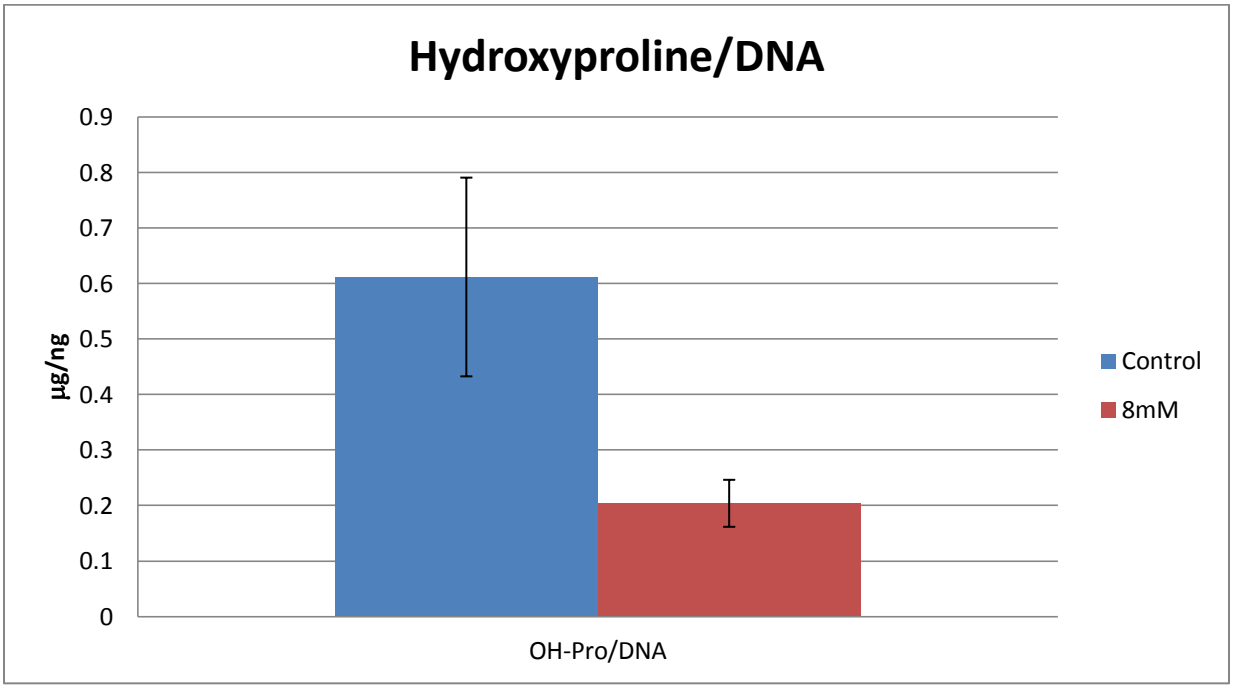


Figure 5. Quantification assays: Sulfated GAG content of pellets normalized to DNA.

Hydroxyproline contents of pellets normalized to DNA.

3.4 Discussion

Histological analyses demonstrated clear differences between control (1.8 mM Ca^{2+}) and elevated 8mM Ca^{2+} experimental pellets, most notably the differences in pellet morphology and IHC collagen staining. The pellets cultured in elevated Ca^{2+} exhibited loose and stratified layers towards the outer portion of the pellet, as opposed to the compacted uniform appearance of control pellets. Hematoxylin staining of cell nuclei indicated a sparse cell population in the loose outer layers of the 8 mM Ca^{2+} pellets, with the majority of cells being distributed towards the center of the pellet. This cell distribution was the opposite of the control pellets, where cell number was higher in the pellet periphery, and diminished towards the center. Immunohistochemistry results for collagen X indicated enhanced collagen X staining within the loose peripheral layers of the elevated Ca^{2+} pellets. Collagen X is generally associated with chondrocyte hypertrophy and ossification and calcified cartilage, where bone and cartilage tissue interact. These results suggest that cells toward the outside of the pellet may have become hypertrophic and possibly apoptotic. This is consistent with previous studies showing the role of PTHrP in inhibiting hypertrophy in chondrocytes[10-13]. Parathyroid hormone related-protein is down-regulated in environments of high Ca^{2+} concentration due to signaling through the extracellular calcium sensing receptor[4, 11]. Increased PTHrP has been associated with increased chondrocyte proliferation and maintenance of cells in a matrix forming phenotype, resulting in increased matrix production[10, 11].

Histological findings demonstrated that both 8 mM and 1.8 mM Ca^{2+} pellets differentiated down the chondrogenic lineage, but to varying degrees. Alcian blue staining showed both conditions stained positive for sulfated GAGs, but pellets cultured in 1.8 mM Ca^{2+} appeared to stain more positively than pellets cultured in 8 mM Ca^{2+}. These qualitative findings were supported by the quantitative results of GAG and DNA quantification assays. When normalized to DNA, control pellets produced nearly twice the sGAGs as elevated Ca^{2+} pellets. Similar results were observed via IHC for collagen II, where both pellets stained positive, but the control pellet stained more intensely than the elevated Ca^{2+} pellets. This was

supported with the results of hydroxyproline quantification. Control pellets produced nearly three times more hydroxyproline than pellets cultured in 8 mM Ca^{2+} when normalized to DNA (p=.09), corresponding to nearly three times the collagen II present within the pellet. At the level of gene expression, aggrecan mRNA expression at day 7 was diminished in hASC cultured in 8mM Ca^{2+}, although the difference was not significant.

In addition to histological and IHC analyses for chondrogenic markers, further analyses were performed to determine the effects of elevated Ca^{2+} on osteogenic differentiation. Pellets in 8 mM Ca^{2+} exhibited positive alizarin red staining, especially towards the center of the pellet, while the 1.8 mM Ca^{2+} controls did not exhibit any positive staining. IHC for collagen I showed both control and experimental pellets stained positively, but in different locations. In elevated Ca^{2+} sections, positive IHC staining was mostly localized to the loose outer layers of the pellet, whereas the control pellets demonstrated positive collagen I staining in a swirled pattern in the center of the pellet. The mechanisms responsible for these positive results are not clear and require further study.

This study has demonstrated that culturing hASC in chondrogenic differentiation medium with elevated 8mM Ca^{2+} results in inhibited hASC chondrogenesis. While chondrogenic differentiation can occur to some extent, the cells closest to the elevated Ca^{2+}environment, towards the outer portions of the pellet, appear to become hypertrophic, and undergo apoptosis, resembling zones of calcified cartilage. Additionally, this process appears to decrease production of cartilage extracellular matrix components. These differences in cell morphology and extracellular matrix production may be indicative of varying mechanical properties within the pellet and this should be further studied. This may prove beneficial in future cartilage tissue engineering studies, as articular cartilage is highly organized and stratified with each layer having different mechanical properties. The outer, superficial layer is mostly acellular, composed primarily of collagen II fibrils and water. Deeper within the tissue, in the

intermediate and deep zones, a sparse community of chondrocytes are surrounded by a network of sulfated GAGs and proteoglycans, with larger collagen II fibrils running perpendicular to the joint through the tide mark into the zone of calcified cartilage. The zone of calcified cartilage is found beneath the tidemark, as the tissue transitions to subchondral bone with the function of anchoring the cartilage tissue to the bone[14-17]. The observed hypertrophy in this study may provide key insights for engineering appropriately layered articular cartilage, with integration of that cartilage to the underlying bone.

3.5 Conclusion

Elevated extracellular calcium has proven effective in modulating chondrogenic differentiation of hASC in pellet culture. While cells are able to chondrogenically differentiate, and produce some cartilage extracellular matrix constituents, they produce less matrix in elevated Ca^{2+} conditions compared to controls, and some measures of osteogenic differentiation are enhanced. Further study must be done to determine the material properties of the resulting tissue, as well as observe changes in the expression of chondrogenic RNA markers through the differentiation process.

Chapter 4:

Targeting the Extracellular Calcium Sensing Receptor in Human Adipose Derived Stem Cell Osteogenic Differentiation

The previous experiment further demonstrated the ability of elevated extracellular Ca^{2+} to impact hASC differentiation, however the mechanism by which elevated calcium directs cells during differentiation is still not clear. This study aims to modulate the effects of elevated calcium by targeting two different proteins. PC2 is a permeable ion channel associated with the primary cilia, in a mechanosensing role. It utilizes Ca^{2+} as a secondary messenger for cell signaling. We will use siRNA to knock down its expression. The extracellular calcium sensing receptor is a G-protein coupled receptor which allosterically binds with Ca^{2+}, allowing it to act as a first messenger. We will modulate it using allosteric binding ligands to activate (calcimimetics) and inactivate (calcilytics) the signaling process.

4.1 Introduction

Calcium plays a key role in cell metabolism and can act as an extracellular signaling molecule, directing a number of cellular processes through various mechanisms. One mechanism is through intra- and extracellular transport of calcium ions through voltage-gated calcium channels, while others require the presence of a ligand to allow ions to travel across the cell membrane[1, 2]. This ion transport can result in cell secretion or contraction activities, directing specific cell activities. A number of ion transport channels are associated with mechanotransduction, allowing the cell to interpret mechanical loads through the movement of calcium ions. Polycystin 2, a sensory protein associated with mechanotransduction functions of primary cilia, is believed to function as a permeable calcium channel.[3]

Calcium can also act as an external first messenger without being transported into the cell. The extracellular calcium sensing receptor (CaR) is a membrane bound G-protein coupled receptor that binds to calcium ions externally, initiating a signaling mechanism internally[4, 5]. This receptor plays a key role in maintaining calcium ion homeostasis in that it signals to the cell the concentration of extracellular calcium, allowing the cell to respond appropriately. The CaR is often associated with the secretion of PTHrP, and has been targeted in pharmacological treatments for a variety of diseases, including osteoporosis. Various ligands have been allosterically bound to the CaR to either activate (calcimimetics) or inactivate (calcilytics) this signaling mechanism[4, 6-10].

Our lab has found that elevating the concentration of extracellular calcium in the culture medium can impact both osteogenic [11] and chondrogenic (unpublished data) differentiation of human adipose derived stem cells (hASC). We have shown that a Ca^{2+} concentration of 8mM is effective in directing hASC osteogenic lineage specification in the absence of other osteogenic factors, while the same concentration appears to inhibit chondrogenic lineage specification when hASC are cultured in chondrogenic differentiation media including soluble chondrogenic growth factors transforming growth factor beta-1 (TGF-β1) and bone morphogenetic protein 6 (BMP6) (unpublished data). The mechanism by which elevated Ca^{2+} influences hASC lineage specification is unclear.

The aim of this study was to determine if the PC2 channel and/or the CaR play a significant role in regulating hASC differentiation in response to elevated extracellular calcium. Understanding the mechanism by which hASC lineage specification is directed by extracellular Ca2+ may lead to significant advances in use of these stem cells for tissue engineering and regenerative medicine applications.

4.2 Materials and Methods

Cell Isolation and Culture

Excess adipose tissue was obtained from five donors (24 to 37 year old females, multiple ethnicities) in accordance with an approved IRB protocol at UNC Chapel Hill (IRB 04-1622). Human ASC were isolated from the tissue using a method described by Zuk et al[12] as previously described by our lab [13, 14]. At passage 2, 100K cells from each donor were seeded in a single flask in complete growth media (CGM) comprised of alpha-modified minimal essential medium (α-MEM)(Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Premium Select, Atlanta Biologicals, Lawrenceville, GA), 200 mM L-glutamine, and 100 I.U. penicillin/ 100 µg streptomycin per ml (Mediatech, Inc.) The cells were allowed to proliferate at 37°C in 5% carbon dioxide until reaching 70% confluency, and then trypsinized. The amassed cells were characterized for multipotent lineage capability, ensuring the amassed cells differentiated representative of an average of the five cell lines.

PC2 siRNA Knockdown

A single cell line of hASC was plated in a 12 well tissue culture plate and allowed to reach 50% confluency. Polycystin 2 was transiently knocked down in the cells using the Invitrogen siRNA protocols. Small interfering RNA specific to PC2 was used for experimental cells, while nonspecific scramble siRNA was used as a knockdown control. Lipofectamine 2000 was used as a transfection reagent to introduce the siRNA into the cells during the knock-down process. Knockdown cells were cultured in complete growth media with elevated (8mM) Ca^{2+}. Calcium levels were elevated to a concentration of 8mM by dissolving $CaCl_2$ into the media. Control cells were cultured in complete growth media with and without elevated Ca^{2+}, and all cells were incubated at 37^{o} in 5% carbon dioxide. At day 7 the knockdown procedure was repeated, and returned to conditioned media at day 8. At day 14, cells from each well were scraped and suspended in .5 N HCl for analysis of calcium and protein. Calcium was quantified using a calcium liquicolor assay (Invitrogen) and a Tecan GENios microplate reader with Magellan 5 software (Tecan, Zurich, Switzerland). Protein quantification was conducted with a BCA protein assay

(Pierce) and the same microplate reader. Calcium values were normalized to protein concentrations for all samples.

Ligand Preparation for Pharmacologic Regulation of CaR

Allosteric binding ligand solutions were prepared by dissolving samples of each chemical (Cinacalcet HCL (Santa Cruz Biotech, calcimimetic), NPS 2143 (Santa Cruz Biotech, calcilytic), Calhex 231 (Santa Cruz Biotech, calcilytic), and AMG 568 (Santa Cruz Biotech, calcimimetic)) in absolute ethanol at a final concentration of 10^{-3} M. Solutions were aliquoted and stored at -20^{o}C.

Ligand Selection

Preliminary experiments were utilized to determine which ligands modulated the effects of elevated extracellular Ca^{2+}. A single cell line of hASC was plated in tissue culture treated plates and allowed to reach 50% confluency. Ligands were combined with media at a final concentration of 10^{-5} M and added to cells. Control cells were treated with 10μl of pure ethanol. Experimental cells and untreated controls were cultured in complete growth media with and without elevated extracellular Ca^{2+} for 14 days receiving media changes treated with ligands every 48-72 hours. At day 14, Cells from each well were scraped and analyzed for Calcium and protein as previously described.

Cell Culture

A superlot of previously characterized premenopausal hASC was plated and allowed to reach 70% confluency prior to receiving experimental treatments. Control cells were then treated with complete growth medium (MEM alpha, L-Glutamate, Pen/strep, and FBS), complete growth medium with elevated Ca^{2+}, and osteogenic differentiation media. Calcium was elevated by dissolving $CaCl_2$ in CGM to raise the Ca^{2+}concentration to 8mM as described above. Experimental cells were cultured in complete growth medium with elevated Ca^{2+}, with allosteric binding ligands added to the media prior to

media changes. Cinacalcet HCL (calcimimetic) was added at a final concentration 10^{-6} M and NPS2143 was added at 5x 10^{-7} M. Control cells were cultured with 1μl of ethanol at each media change. Cells were cultured at 37°C in 5% carbon dioxide for up to 14 days, receiving media changes every 48-72 hours.

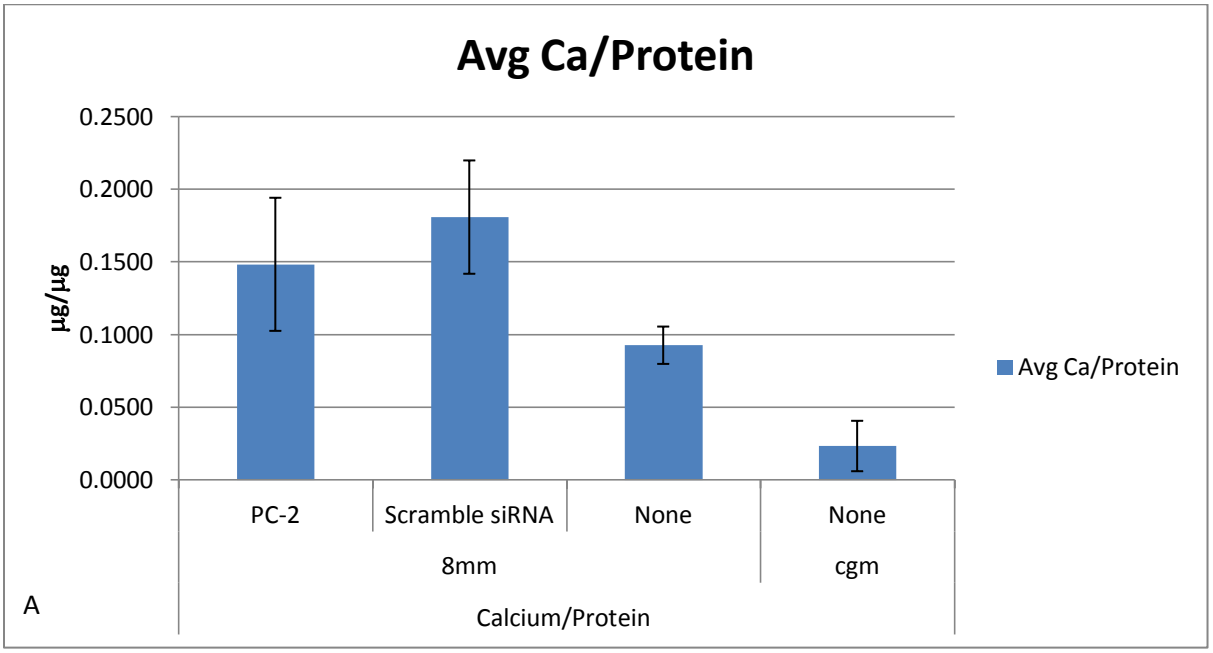
Real time PCR

Cells from each experimental condition were collected on days 3, 9, and 12 of culture for real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Each construct was dissolved in RNA lysis buffer and total RNA was isolated using a Perfect RNA Eukaryotic Mini kit (Eppendorf, Westbury, NY). Complementary DNA was synthesized using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA) with oligo $(dt)_{20}$ primers. Primers and probes for human Runx2 (Assay HS00231692 M1), BMP2 (Assay, HS00154192 M1), Osteopontin (SPPI, Assay HS2408577 M1), Alkaline Phosphatase (ALP, Assay HS01029144 M1), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH, Assay HS99999905 M1) were purchased from Assays-on-demand (Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed using TaqMan PCR Master Mix (Applied Biosystems) in an ABI Prism 7000 system (Applied Biosystems). Signals were normalized to GAPDH expression levels using the $^{-\Delta\Delta CT}$ method [15].

4.3 Results

Knockdown of PC2 did not result in significantly different cell mediated calcium accretion from scramble siRNA controls (Fig 1a). Cells treated with CaR binding ligands in CGM at basal calcium levels (1.8 mM) did not demonstrate significant differences in proliferation or calcium to protein ratios (not shown). However, hASC treated with allosteric binding ligands and cultured in elevated 8 mM Ca^{2+} demonstrated significantly different calcium to protein ratios. Cells treated with either calcilytic (NPS2143, Calhex 231, Santa Cruz Bio) exhibited lower calcium to protein ratios, while cells treated with

either calcimimetic (R568, Cinacalcet HCL, Santa Cruz Bio) exhibited higher calcium to protein ratios. (Fig 1b)



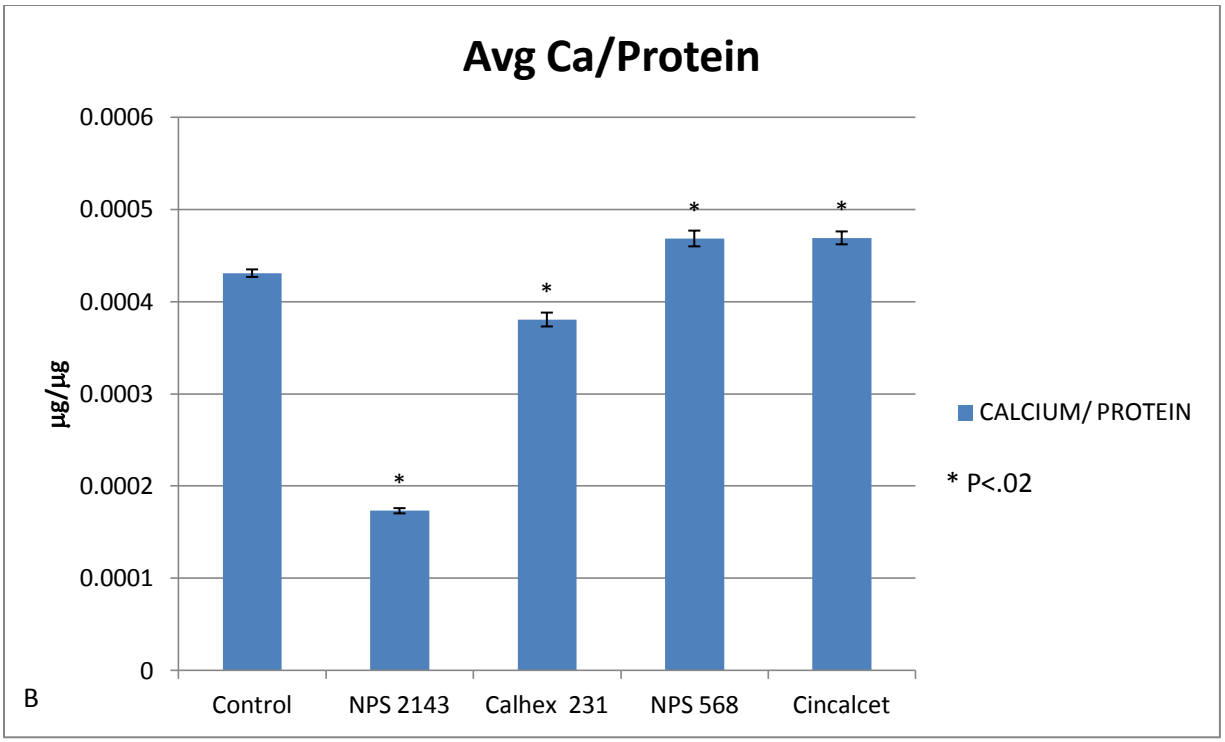


Figure1. Calcium to protein ratios: A) Transient knockdown of PC2 B) Allosteric binding of CaR in elevated calcium

Assays

All cells cultured in CGM with 8mm Ca^{2+} demonstrated significantly higher calcium to protein ratios when compared to untreated controls cultured in CGM with basal levels (1.8 mM) of Ca^{2+}(Fig 2). Human ASC cultured in ODM or in the presence of 8 mM Ca^{2+} and combined with the calcimimetic Cinacalcet exhibited significantly higher calcium to protein ratios when compared to untreated controls cultured in CGM with 8mM Ca^{2+} without Cinacalcet (Fig 2). However, culture with the calcilytic NPS 2143 produced inconsistent results at various stages and often resulted in cell death.

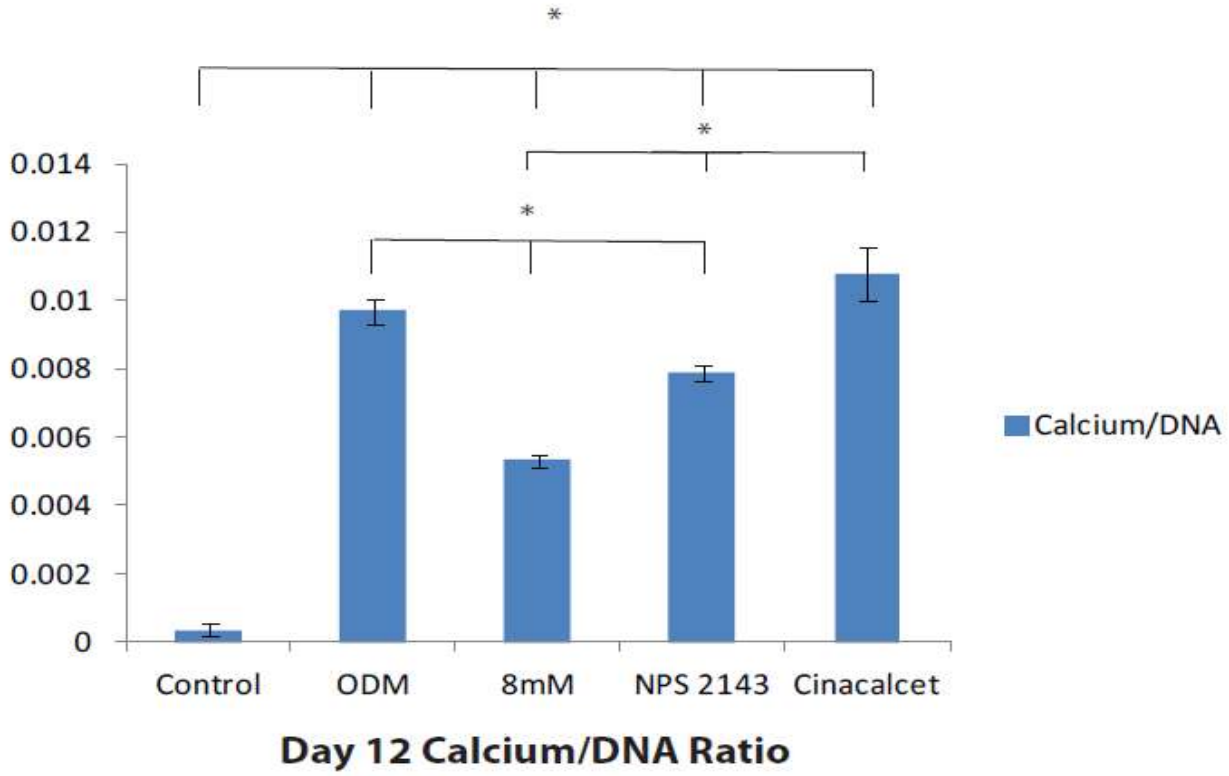


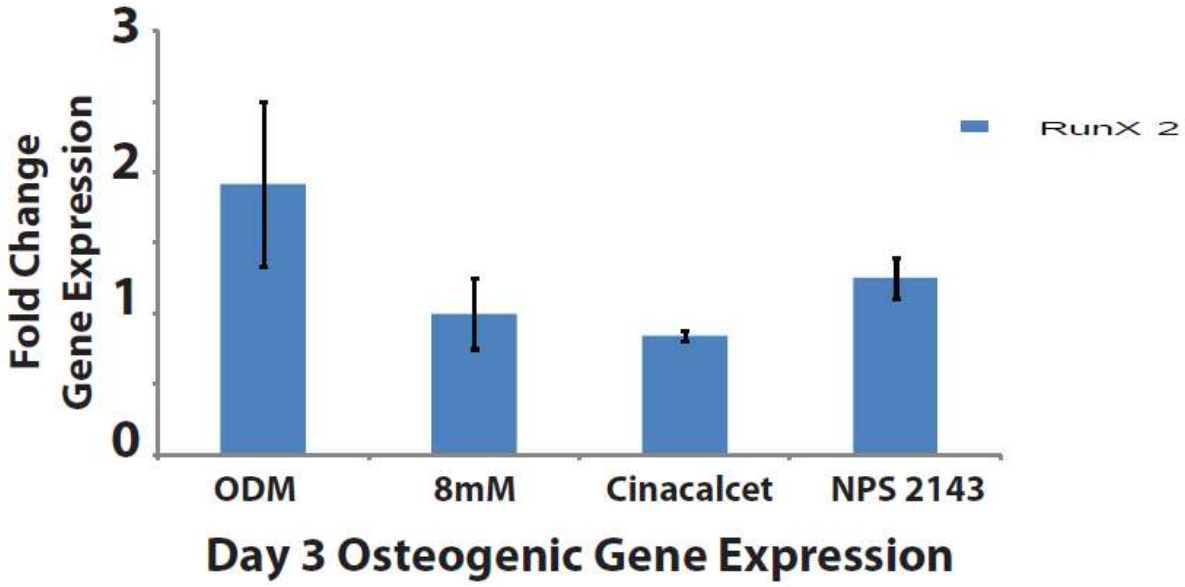
Figure 2.Calcium to protein ratio at Day 12

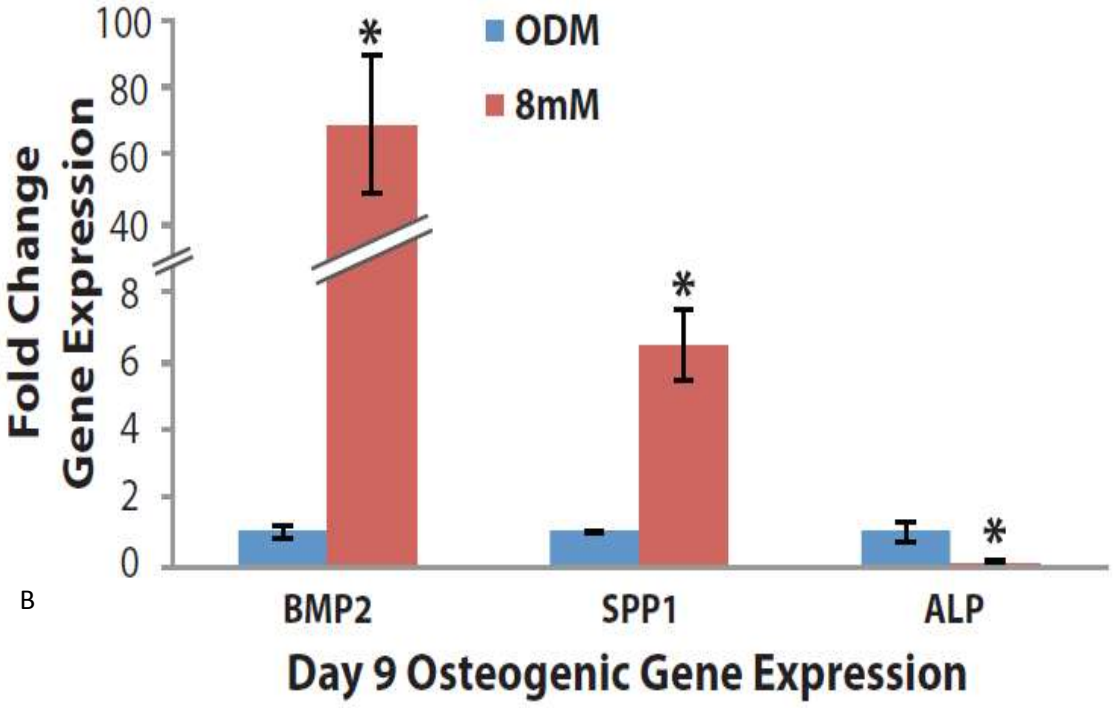
RT-PCR

PCR analysis revealed differences in mRNA expression at various time points in each of the conditions. The comparison between cells cultured in osteogenic differentiation medium and complete growth medium with 8 mM Ca^{2+} at day 9 showed significant differences in osteogenic markers at this time point (Fig 3b). Cells cultured in CGM with 8mM Ca^{2+} had a nearly 7 fold increase in osteopontin mRNA expression compared to cells treated with ODM, and a greater than 60-fold increase in BMP2 mRNA expression (Fig 3b). Alkaline phosphatase mRNA expression, however, was significantly down regulated at this time point. Expression of the early osteogenic differentiation marker RUNX2 was compared at day 3 for cells cultured in ODM, CGM with 8mM Ca^{2+}, and cells treated with Cinacalcet. There was no significant difference in Runx2 mRNA expression among any of the samples (Fig 3a). At

day 12 cells cultured in CGM with 8 mM Ca^{2+} expressed alkaline phosphatase significantly greater than that of hASC cultured in ODM and hASC treated with Cinacalcet (Fig 3c). Additionally, hASC cultured in CGM with 8mM Ca^{2+} expressed significantly higher levels of osteopontin than cells cultured in ODM, yet lower than hASC in CGM with 8mM Ca^{2+} and further treated with Cinacalcet (Fig 3c).

A





B

Figure 3. qPCR results: A) Day 3 Runx 2 expression B) Day 9 BMP2, ALP, and SPPI expression

C

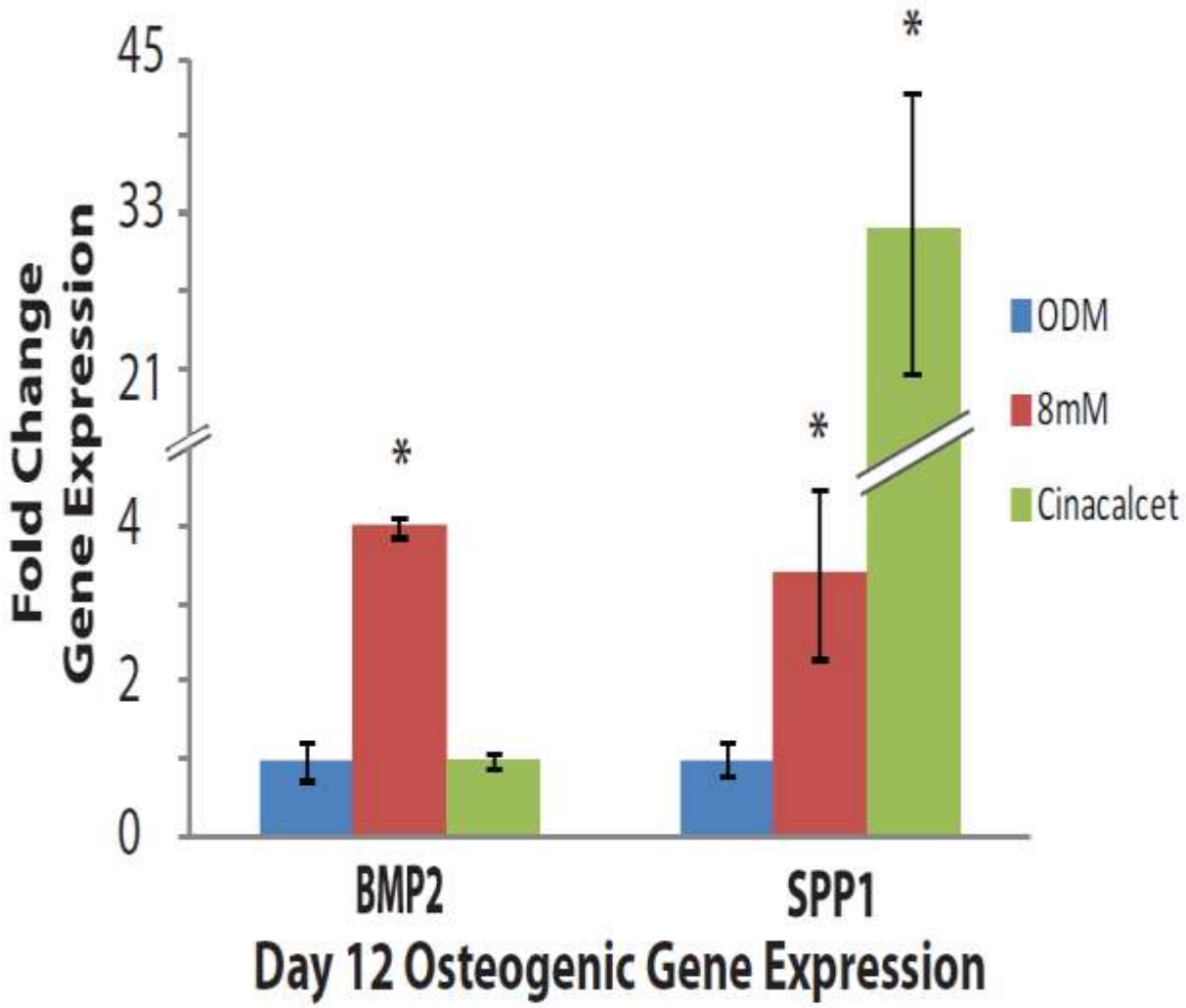


Figure 3. qPCR results C) Day 12 ALP and SPPI expression

4.4 Discussion

We chose to utilize NPS2143 as the calcilytic based on preliminary results indicating its significant effect on diminishing osteogenic differentiation of hASC in response to 8mM Ca^{2+} (fig1b). However, after extensive repeat testing, this compound produced inconsistent results in hASC response, including cell death at various concentrations and time points that had been previously tested with no

negative effects. We believe the inconsistency in hASC response to the ligand was potentially a result of inconsistencies in the chemical as received from the manufacturer as cell responses from same donors varied with different samples of the same compound purchased at different dates. Due to these variations, we will not conclusively state that addition of a calcilytic that inhibits CaR activity results in diminished hASC osteogenic response, although our preliminary analyses indicate that it might.

Our study demonstrates that elevating extracellular Ca^{2+} in complete growth medium to a concentration of 8mM is effective in directing hASC osteogenic differentiation similar to culture in traditional osteogenic differentiation medium as demonstrated by cell mediated calcium accretion and the expression of osteogenic markers. At day 3 the two conditions are not significantly different in mRNA expression of Runx 2. At Day 9, hASC cultured in CGM with 8 mM Ca^{2+} express BMP2 and osteopontin mRNA at significantly greater levels than cells cultured in ODM, but ALP mRNA expression is significantly less. By day 12 however, hASC cultured in 8mM Ca^{2+} exhibited significantly higher levels of ALP mRNA expression compared to hASC cultured in ODM, with little change in the difference of SPP1 expression. BMP2 is often associated with the early phases of osteogenesis, while osteopontin, associated with mineralization is known to peak twice during the differentiation process, around Day 4 and again around day 14 during mineralization[7, 20]. Osteogenic differentiation of mesenchymal stem cells generally occurs in three phases: proliferation, matrix maturation, and mineralization, with varying gene expression at different time points of the process[16, 17]. Findings from our study suggest that while both ODM and 8mM Ca^{2+} culture are effective in inducing hASC osteogenic lineage specification, the elevated Ca^{2+} and lack of certain chemical cues may cause cells to change their pattern of gene expression, and the length of time needed to fully differentiate and produce a mineralized matrix. The osteopontin levels at day 12 demonstrate that cells cultured in 8 mM Ca^{2+} were still upregulating mineralization processes; however cells cultured in ODM had higher calcium to protein ratio at the same time point. This suggests that given a longer culture time cells may have produced similar calcium to protein ratios.

This is consistent with other studies that have investigated the role of elevated extracellular Ca^{2+} on osteogenic differentiation of other cells[18, 19]. An *et al*[19] recently showed that elevated Ca^{2+} increased mineralized matrix nodule formation in human dental pulp cells. They also reported increased expression of osteopontin and diminished alkaline phosphatase expression, consistent with findings from this study.

While the calcilytic NPS 2143 was inconsistent in effectively modulating cell activity, the calcimimetic Cinacalcet performed as expected by enhancing hASC osteogenic differentiation. The calcium to protein ratio observed in treated cells at day 12 was similar to that observed in cells cultured in ODM, however, as discussed earlier, the various media types resulted in differentiation at different rates, with different time points for gene expression. These findings suggest that a longer culture period may produce significant differences in mineralized matrix production, especially considering the significantly upregulated SPPI expression at day 12 in Cinacalcet treated cells. Overall, cells cultured with Cinacalcet in 8mM Ca^{2+} behaved similarly to cells cultured in ODM throughout the experiment, with the exception of the increased osteopontin expression at day twelve. These data suggest that the addition of Cinacalcet to CGM with 8mM Ca^{2+} causes hASC osteogenic lineage specification similar to ODM, with an enhanced mineralization potential.

These results suggest that modulation of the CaR is more effective than PC2 knockdown in affecting hASC osteogenic differentiation, but only in the presence of elevated extracellular Ca^{2+}. This may demonstrate that impact of elevated calcium ions on hASC differentiation may be more attributed to their role as external first messengers rather than transport or secondary messaging. Jensen et al similarly demonstrated that elevated Ca^{2+} as low as 2.5 mM was effective in inhibiting adipogenesis of preadipocytes[21]. Yet in their study cells that did not express CaR did not exhibit the effects of elevated Ca^{2+} on adipogenesis, suggesting CaR, alone, was responsible for the effects of elevated Ca^{2+} on

differentiation[21]. CaR was also shown as a key mechanism with elevated Ca^{2+} in human keratinocyte differentiation. Tu et al demonstrated how inhibiting CaR negatively affected cell differentiation, and proliferation of human keratinocytes[20]. That study utilized antisense RNA to inhibit CaR similar to our early use of siRNA to knock down PC2, whereas our study used allosteric ligand binding to inactivate CaR signaling. Future studies may compare the effectiveness of modulating CaR activity using transient knock down via siRNA, and allosteric binding, with the ultimate goal of optimizing stem cell differentiation. Additional studies must determine the unintended impacts of each treatment on cell culture and differentiation. Specifically, we must determine how transfection impacts proliferation, and cell viability, as well as ensuring ligands are in appropriate concentration to be effective without becoming toxic. Both may affect gene expression during the osteogenic differentiation process.

4.5 Conclusion

This study has shown the effectiveness of using 8mM Ca^{2+}as a first messenger directing hASC differentiation down an osteogenic lineage, through modulation of the extracellular calcium sensing receptor. Targeting the CaR to regulate hASC differentiation may be an effective method of optimizing conditions for tissue engineering and regenerative medicine applications using hASC. More studies must be done to better understand the role of CaR signaling in hASC lineage specification. In particular, we must determine the best method to both activate and inactivate the CaR for extended durations, and determine how CaR modulation affects hASC fate.

Chapter 5:

Conclusions

5.1 Conclusions

This body of work has focused on various factors affecting chondrogenic differentiation of hASC with the ultimate goal of optimizing conditions for engineering functional cartilage tissue. The use of hASC in novel treatments for cartilage defects is inevitable, and the application of the work presented here should greatly affect that emerging field.

The first focus of this work was on the conditions needed to optimize chondrogenic differentiation of hASC, specifically, the appropriate mechanical loading environment. Like hMSC, hASC best differentiate when cultured in a three dimensional environment, under hypoxic conditions, cultured in media containing insulin, high glucose, dexamethasone, ascorbic acid, and soluble growth factors TGF-β3 and BMP6. Additionally, compression and hydrostatic pressure at magnitudes encountered in vivo seem best suited to induce chondrogenic differentiation, though fluid shear and tensile strain each play a role in cartilage tissue homeostasis and could be incorporated in conditioning engineered tissue.

The mechanisms by which these mechanical loads signal hASC differentiation are not fully understood, but are associated with the elevation of intracellular calcium concentration, which directs cell activity. Intracellular Ca^{2+} is elevated by two mechanisms: transport through ion channels into the cell, or signaling the release of calcium stores from the endoplasmic reticulum into the cytoplasm. Some Ca^{2+} channels are activated by mechanical load along the plain of the membrane, allowing Ca^{2+} influx as a result of shear and tensile strain [3]. Other studies have shown the role of substrate rigidity, and RhoA

pathways in directing intracellular Ca^{2+} concentration, and ultimately stem cell differentiation [1]. This demonstrates the importance of Ca^{2+} in mechanical signaling. Additionally, elevating extracellular Ca^{2+} concentration has been shown to increase osteogenic differentiation in hASC, as well as decreased adipogenesis in preadipocytes [2,4]. In this study, we observed the impact of elevated Ca^{2+} on chondrogenic differentiation of hASC in pellet culture. Pellets cultured in elevated 8mM Ca^{2+} produced less cartilage products than control pellets suggesting the elevated Ca^{2+} inhibited chondrogenic differentiation. Histological analyses demonstrated the outer layers of experimental pellets experienced terminal differentiation of chondrogenic cells, resulting in hypertrophic calcified cartilage. The zone of calcified cartilage plays a key role in anchoring cartilage tissue into place. Engineering cartilage tissue with a functional anchoring layer may introduce new methods of implantation and improved integration into native tissue. The observed results are significant in that they may provide insight on engineering cartilage tissue with each layer seen in vivo, improving the functionality of engineered tissue.

Recognizing the effect of elevated Ca^{2+} on chondrogenic, adipogenic, and osteogenic differentiation of hASC, further study on the mechanism by which elevated calcium signals cells was conducted. Preliminary work targeted PC2 with siRNA, and did not effectively modulate the effect of elevated calcium on hASC differentiation. Additional work targeted the CaR, which allows calcium to act as a first messenger, signaling cell processes without crossing the cell membrane. This was accomplished using both calcilytics to inactivate the CaR, and calcimimetics to activate the CaR. These allosterically bound ligands were effective in modulating the impact of elevated Ca^{2+} on hASC differentiation. Upon further study on the impact of the ligands, gene expression with osteogenic differentiation medium and growth media with elevated calcium was compared. Cinacalcet HCL, the calcimimetic studied was effective in enhancing osteogenic differentiation in hASC with gene expression similar to that of ODM. Additionally, markers for mineralization were upregulated in cells treated with Cinacalcet at the end of the culture period, suggesting a greater capacity for mineralization. This study demonstrates the impact

of elevated Ca^{2+} on hASC differentiation may have more to do with signaling pathways, than ion transport. Additionally, it demonstrates that the CaR is an effective target to modulate hASC differentiation, and can be used to optimize tissue engineering.

5.2 Future Work.

Additional work with hASC pellets cultured in chondrogenic media with elevated Ca^{2+} may be in order to determine the material properties of the matrix products created. Further, these pellets should be analyzed to determine if the layers observed are cohesive in a manner similar to that of articular cartilage. This may allow for the development of engineered cartilage tissue designed to be anchored on the subchondral bone, allowing for better assimilation.

The calcilytic NPS2143 was not consistent in inhibiting CaR without causing cell death. Future studies to inhibit CaR may require a different calcylitic or the use of siRNA to transiently knock down CaR. Also, a study to determine the optimal time to modulate CaR in engineering various tissues may prove useful for future design protocols.

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Chapter 2

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